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THE CELL BIOLOGY OF HYDRA

THOMAS L. LENTZ

1964

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THE CELL BIOLOGY OF HYDRA

Thomas Lawrence Lentz

A Thesis Presented to the Faculty of
the Yale University School of Medicine
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Medicine

Department of Anatomy
Yale University School of Medicine
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Introduction

These studies represent an investigation of several aspects of the cellular biology of hydra. This work began as a study of the enzyme histochemistry of hydra. The results of these experiments demonstrated remarkable intracellular and regional localizations of enzyme activity and suggested that this animal could be used to study the relationship of enzymes to a variety of cell functions. Following the establishment of a histochemical baseline, morphological investigations were carried out utilizing the electron microscope. In addition, the relationship of enzymes to permeability and the function of specialized effector cells and enzymatic changes during differentiation and regeneration were studied.

As these studies proceeded, it became evident that the hydra illustrated most of the processes included in the general category of development, including cell division, growth and differentiation, morphogenesis, induction, polarity, and regional specialization. Furthermore, it became apparent that the hydra possessed a system for the rigorous and precise control of these processes. A major portion of this work concerns the investigation of the factors responsible for the control and organization of growth, differentiation, and anatomical

form of this animal.

The various studies are presented in individual chapters. The first chapter presents a translation and a commentary on the researches of Abraham Trembley, the first to study hydra and also the first to carry out experimental studies on regeneration, budding, and grafting. His writings, in addition, include excellent observations of the habits and behavior of hydra. The second chapter, also introductory, reviews the structure and biology of hydra with special emphasis on development and the theories of development that have arisen from studies on hydra. Following these introductory chapters, the various experimental studies are presented; namely, enzyme and amine histochemistry, nematocyst discharge, enzymatic changes during regeneration, the role of the nervous system in regeneration, fine structural morphology, and permeability. The last chapter is a summary containing the most important findings and conclusions drawn from these studies.

Fig. 1 Abraham Trembley (1710-1784)



Chapter 1

The Observations and Experiments of Abraham Trembley

The fresh water hydra has held exceptional value for the investigation of biological phenomena since its discovery by Leeuwenhoek in 1702. The last decade, especially, has seen a resurgence in the use of hydra as a tool in biological research and as a type useful in classroom instruction. In view of the present knowledge of the biology of this animal, it may seem unusual for investigators to turn back to the pioneer studies on hydra performed over two hundred years ago. And yet the researches of a Swiss tutor, Abraham Trembley (1710-1784) (Fig. 1), continue to be a valuable source of information to those working on this animal today. Trembley, who was one of the first to employ an experimental approach in the field of zoology, published his findings in his Mémoires in 1744. Some of the important experiments described in the Mémoires include the first controlled experiments on regeneration and grafting.

Although Trembley's life has been completely described by Baker ('52), a brief biography is presented here. Abraham Trembley was born at Geneva on September 3, 1710, the son of Jean Trembley. As a young man, he studied mathematics at the academy founded by Calvin and successfully defended his thesis on infinitesimal calculus

in 1731. Trembley went to Holland in 1733 where, in Leiden, he met William Bontinck, a Curator of the University of Leiden, who employed him as tutor to his two young sons. It was during his stay with Count Bontinck, at the mansion of Sorgvliet outside the Hague, that Trembley was to perform his most notable scientific experiments. Since Bontinck's two sons were too young to receive instruction, Trembley was permitted to spend two years at Varel in Oldenburg as tutor to the Prince of Hesse-Hamburg. At Varel, in 1739, Trembley undertook his scientific activities. His early endeavors were not significant, but were invaluable to Trembley in that they led to the beginning of his correspondence with René-Antoine Réaumur which lasted until Réaumur's death in 1757. Réaumur was generous in the assistance, encouragement, and friendship he bestowed upon Trembley. The letters (M. Trembley, '43) illustrate how Réaumur was able to establish firmly the study of animals for their inherent interest without regard to practical application.

Trembley returned to Sorgvliet in 1739 where, the following year, he carried out his first significant scientific research, the confirmation of Bonnet's work on parthenogenesis in lice. Shortly afterward, Trembley discovered the green hydra, Chlorohydra viridissima. He devoted most of his attention to the hydra in the following years and published his findings in 1744 (Fig. 2). Besides these researches, Trembley during the years 1741 to

1746 at Sorgvliet made other important discoveries concerned mainly with colony formation and budding in both the protozoa and the metazoa.

Trembley was a friend and tutor in the Bentinck family until 1747, when his pupils entered the University of Leiden. He then journeyed to England and became closely associated with the influential Duke of Richmond. The years until 1757 were largely spent in travel to all parts of Europe. Trembley sent his last scientific publication for eighteen years to the Royal Society in 1757.

Trembley was married at the age of 46 in 1757 to Mademoiselle von der Strassen. He and his young bride settled down in a country house near Geneva where the following years were spent in the rearing of five children and in political activities in Geneva. Trembley had evolved a system of education for his children which was published in 1775. Many of his scientific observations were recorded in this book but have been largely overlooked, since the book was addressed to children. The most significant observation contained in this work was that of the process of cell division in diatoms. This discovery was also published by Bonnet in 1779. Although Trembley did not realize the significance of this observation, his sketches unmistakably illustrate the process of cell division and are probably the first to do so.

During the latter part of his life, Trembley devoted less time to scientific pursuits and became absorbed

in politics and religion. In the spring of 1784, when he was engaged with his last work, his strength declined and he lapsed into an illness from which he never recovered.

The hydra was not entirely unknown before Trembley began his observations, as Leeuwenhoek had discovered and briefly described the animal some forty years earlier (Leeuwenhoek, 1702, 1706, 1714). He related his discovery in the Philosophical Transactions: "Moreover, I observed one of these animalcula, whose body was sometimes long, and sometimes contracted, and about the middle of its body, which I conceived to be the lower part of its belly, there was another of the same kind, but smaller, the tail of which seemed to be fastened to the other. I caused one of these animalcula (for the sake of its wonderful figure and generation) to be drawn by my limner about twice as big as it appeared to the naked eye, whilst it was in the water, and fast linked to the root of the aforesaid green weeds..... The horns appeared of so wonderful a make to our eyes, that it almost puzzled the limner to draw them....." (1702, p. 1305).

Shortly after Leeuwenhoek had made his announcement, a mysterious gentleman of the country reporting to the Royal Society (Anon., 1703) through Mr. C. described an animal discovered in some water taken out of a ditch. Mr. C. recognized the animal to be the same as that discovered by Leeuwenhoek and confirmed the observation of the latter.

These initial descriptions concerned the brown hydra, of which there are several species. Trembley, however, was the first to describe the green hydra, Chlorohydra viridissima. He relates this event in his Mémoires.

"Ever since the first summer 1740 that I passed at Sorgvliet, the country house of Count Bentinck situated a quarter of a league from The Hague, I have found polyps there. Having noticed various small animals on the plants that I had taken from a ditch, I put some of the plants in a large glass full of water, and this I placed on the inner sill of a window; I then occupied myself in studying the insects that were contained in it. I soon saw a large number of them in it, some common kinds in fact, but the majority were unknown to me. So novel a spectacle was presented by these little animals that my curiosity was excited. On running my eyes over this glass so teeming with insects, I noticed a polyp that was fixed on the stalk of an aquatic plant. I did not at first take much notice of it: I followed the little insects that were better adapted by their vivacity to attract my attention than an immobile object; for when one only looks at it casually, such an object could scarcely avoid being taken for a plant, particularly by someone who had no idea of animals approaching these fresh-water polyps in shape, as do the polyps of the sea.

"I have said that the polyp I had noticed was

immobile. It was not that it could not move, but I did not know anything of this at that time.

"Before going any farther, I ought to give here a general description of the form of these animals, so that I can make myself understood. The body ab (Fig. 3) is rather slender. From one of its extremities a project the horns ac, which serve as feet and arms and are even more slender than the body. I call the extremity a anterior, because the head of the polyp is there, and I give the name of posterior to the opposite extremity b....."(1744, p. 7 - 8).

The question now arose concerning the nature of the organism: was it plant or animal? Trembley's initial impression based on the polyp's shape, immobility, and green color was that they were plants. Then he noticed the movement of the arms, bending and twisting slowly in different directions, but still believing that the polyps were plants, he could not suppose that the movements of the polyps were their own. However, the more Trembley studied the movement of the arms on later occasions, the more it seemed that this ability was due to an internal cause and not an impulse external to the polyps. Trembley wrote:

"One day I moved the glass containing them ever so little, to see what effect the resulting movement of the water would have on their arms. I could by no means anticipate the effect it produced. Instead of seeing,

as I had expected, the arms and even the bodies of the polyps simply agitated in the water, and carried along by its movement, I saw them contract suddenly, and so rigorously, that the body of each polyp appeared as a grain of green matter, and each of the arms disappeared entirely from my sight. I was surprised, and this surprise only helped to excite my curiosity, and to redouble my attention. As I examined, aided by a magnifying glass, several of the polyps that I had caused to contract, I soon saw that some of them were beginning to extend: their arms became visible again, and little by little these polyps resumed their original form.

"This contraction of the polyps, and all the movements that I saw them make as they extended themselves again, greatly aroused in my mind the idea of an animal. I compared them at first to snails, and to other animals that contract and expand" (1744, p. 9 - 10).

However, Trembley still was not convinced of the animal nature of the polyps because of their shape and color, and thought them possibly to be a type of sensitive plant. Several days after he made the observations on the contraction and expansion of the polyps, he found several polyps fixed against the side of the glass at a place where he had not seen them before. He soon determined the manner in which they had come there as he was observing them. This observation of their progressive movement succeeded in persuading Trembley that the polyps were indeed animals.

Among the first observations that Trembley made was that individuals of the same species did not have the same number of arms. Although this did not seem unnatural to him, he at first compared the arms of the polyps to the branches and roots of plants as the number varies greatly among the individuals of the same species. The thought that the organisms that he was observing were plants again passed through his mind. It gave impetus to the idea of cutting the polyps because, as Trembley reasoned, if the two parts of the same polyp lived after having been separated and each became a perfect polyp, it would be evident that these organisms were plants. Having been previously convinced that the polyps were animals, he did not attach much importance to the experiment and fully expected to see the cut polyps die.

Trembley cut the first polyp on November 25, 1740 and put the two parts into a flat glass in order to observe the polyps with a lens. He wrote:

"The instant I cut the polyp, the two parts contracted, so that at first they appeared like two small grains of green matter at the bottom of the glass in which I put them. The two parts expanded the same day that I separated them. They were very easy to distinguish from one another. The first had its anterior end adorned with the fine threads which serve as legs and arms of polyps, and the second had none" (1744, p. 13).

Although he observed the first part expand, move its arms, and walk and the second part extend and contract when the glass was moved, Trembley regarded the movement of these two pieces of the same polyp as signs of a feeble remnant of life. He further supposed that since the anterior end contained the head, it would seem natural enough that this part, together with a portion of the body, could survive while the second part, seeming to be a sort of tail, could not live long when separated from the rest of the body. Trembley thus concentrated his observations on this second part to find out how long it would conserve a remnant of life, little realizing that he was about to witness a marvel of regeneration - the growth of a new head on the bottom half of the polyp!

"I observed the portions of the polyp with a magnifying glass several times each day. On the 4th of December, that is to say, the ninth day after having cut the polyp, I seemed in the morning to perceive on the edges of the anterior end of the second part, that which had neither head nor arms, three little points emerging from those edges. They immediately made me think of the horns that serve as the legs and the arms of the polyps. These points were precisely where the arms should be, if this second part had been a complete polyp. Nevertheless, I did not want to decide at once that these were actually arms that were beginning to grow. Throughout the day I

continually observed these points: it excited me greatly, and I awaited with impatience the moment when I should clearly know what they were. Finally, the next day they were so large, that there was no longer any room for doubt that they were actually arms growing at the anterior edge of this second part. The following day, two new arms began to grow out, and a few days later three more. The second part thus had eight of them, which were all in a short time as long as those of the first part.

"I then no longer found any difference between the second part, and a polyp that has never been cut. I had remarked the same thing with regard to the first part, since the day following the operation. When I observed them with the magnifying glass with all the attention of which I was capable, each of the two appeared perceptibly to be a complete polyp, and they performed all the functions that were known to me: they extended, contracted, and walked" (Fig. 4) (1744, p. 15).

Prior to Trembley's work, only two experimental studies on regeneration had been carried out. Réaumur had studied the regeneration of appendages of crayfish (1714) and Thevenot had observed the tail regeneration of the lizard (Réaumur, 1714). Trembley's experiments, however, generated great interest because they were easily reproducible and spectacular in that over half an organism instead of a small portion was capable of regenerating.

Following Trembley's work, Bonnet experimented on aquatic worms (1745) and Spallanzani observed regeneration of various parts of earthworms, tadpoles, salamanders, and snails (1768).

After his first experiment on the division of hydra, Trembley repeated the operation many times and gave precise instructions on the procedure. He wrote:

"In whatever place the body of a polypus is cut, whether in the middle, or more or less near the head, or the posterior part, the experiment has always the same success.

"The animal is too small to be cut at the same time into a great number of parts; I therefore did it successively. I first cut a polypus into four parts, and let them grow; next, I cut those quarters again; and at this rate I proceeded, til I had made 50 out of one single one: and here I stopped, for there would have been no end of the experiment.

"If a polypus is cut lengthways beginning at the head, and the section is not carried quite through; the result is a polypus with two bodies, two heads, and one tail. Some of those bodies and heads may again be cut lengthways soon after. In this manner I have produced a polypus that had seven bodies, as many heads, and one tail (Fig. 5). I afterwards, at once, cut off the seven heads of this new hydra: seven others grew again; and the

heads that were cut off became each a complete polypus" (1744, p. 301).

Thus, in comparing his many-headed monster to the Hydre of Lerne, Trembley himself was the first to use the name by which these animals are most familiarly known today.

Trembley next attempted to obtain union of two animals to produce a single organism. He was able to achieve the grafting of two individuals by impaling a hydra on a boar's bristle and forcing it through the mouth of another specimen. The head end of the inner hydra separated off from the graft but the posterior end remained attached leaving a hydra with two bases (Fig. 6). He then performed a variety of successful grafts involving various portions of hydra. He even attempted to graft different species, but these experiments were unsuccessful. Permanent grafts between two species of hydra with cellular exchange were first achieved in 1960 by Burnett and Lentz (unpublished).

In addition to performing the first controlled experiments on regeneration and the first successful animal grafts, Abraham Trembley recorded thorough proof of asexual reproduction by budding (Fig. 7). His Third *Mémoire* of 1744 concerns the propagation of the polyps.

"After having assured myself, that the fresh water polyps, which are the subject of these *Mémoires*, are able

to multiply by budding, I was extremely curious to know the manner by which they multiply naturally. I was still uncertain, at that time, whether the polyps were plants or animals, and I even flattered myself, as I have said, that their natural manner of multiplying would provide me with a distinctive character of relieving me of my doubt.....

"It was in the month of December, 1740, that I began to look for what was the natural method of multiplication of the polyps. During that month, and during January and a part of February 1741, I observed nothing that gave me cause to flatter myself that I should succeed in satisfying my curiosity on the subject. I could scarcely count on that in winter, but I awaited with impatience the time favorable to the vegetation of plants and the multiplication of animals. The time when I should find what I was looking for came even sooner than I had hoped. On February 25th, I observed with a magnifying glass a green polyp that was fixed to the side of the glass in which I kept all those that were not being in some particular experiment. I noticed a little dark green excrescence on its body. This excrescence, though small, attracted all of my attention for I had never seen anything like it, although I had already observed quite a number. The polyp bearing it was from that moment very precious to me; and for fear of confusing it with several others in the same glass, I took it out and put it by itself in another, It attached itself

to the side of the glass; and by luck it placed itself in such a position that I could easily observe with a strong magnifying glass the excrescence which had appeared on its body. On the same day that I discovered it, after having examined it attentively at different times, I found some resemblance between its structure and the polyp from which it arose. It is true that I could not find any sure conclusion on such an uncertain resemblance, but I started to suspect that this excrescence would become a polyp. The resemblance of polyps fixed on one another which I had seen the previous summer, came back to my mind. It was this especially that made me think that the excrescence which I had observed was a young polyp arising from another. I ceased my observations of February 25th at ten-thirty in the evening, not without impatience to again see the polyp that had occupied me during a part of that day.

"The next morning I had the pleasure of seeing that the excrescence had increased in length; it was about a quarter of a ligne [2.3 mm] in length. It was nearly cylindrical, and situated perpendicularly on the polyp. It became noticeably larger on the 26th and 27th. On the 28th, it was at least half a ligne long. When I caused the polyp to contract, the excrescence contracted also. At last, everything conspired to persuade me that the excrescence was a young polyp, and to be sure of this, nothing was lacking except to see arising from the top of it

the delicate threads that I had named the legs and the arms of the polyps. I continually looked for them; and this same day, February 28th, at ten o'clock in the evening, I saw four of them beginning to push forth. It was not without obvious pleasure that I noticed them, nor without an extreme curiosity to see the end of a phenomenon so new to me. During the following days the four arms of the young polyp elongated and there appeared a fifth. The polyp itself grew noticeably. I saw it extend to the length of at least three lignes while it still held to its mother. It separated on March 18th, between ten and eleven o'clock in the morning" (1744, p. 149 - 152).

The scientific reaction to Trembley's discoveries can be traced in a series of letters to the Royal Society. The first account given to the Royal Society was in a letter from George Louis Leclerc, Comte de Buffon, to Martin Folkes, President of the society, read on October 29, 1741. J. F. Gronovius, an M. D. at Leiden, provided support for Trembley's work in a letter read to the society on November 18, 1742. In his communication he states:

"But of what sort this insect is, is not known; nor have its mouth, stomach, or intestines been yet discovered.

"But of what is most surprising is, that, cut this animal in 5 or 6 pieces, in a few hours there will be as many like their parent.

"This discovery was and is very surprising to all our virtuosos, and really not believed, until the professors, Albinus and Mussenbrock were provided with the animals, and, after having well examined this creature, found the prodigy of increasing itself in that wonderful manner, very true.

"One of the gentlemen that made this discovery was Mr. Allemand, a man of great learning and ingenuity, tutor to the sons of Mr. s'Gravensande" (1742, p. 218 - 219).

W. Bentinck (1743, p. 1 - 2) provided additional confirmation at the request of Folkes, stating that he had seen the experiments "repeated above 20 times". Following this letter, Trembley's paper (1743) on the hydra, translated into English, was read. Réaumur then presented an abstract on the same day in which he said, "M. Réaumur observes, that though in the histories he has already given of minute animals in this work [Sixth volume of M. Réaumur's History of Insects (1734-1742))], he has had occasion to produce many new and unexpected phenomena: one he has now to mention would exceed all belief, if it was not confirmed by the strongest repeated observations, which is, that there are species of insects, who are multiplied by being cut to pieces, and among which, one single animal, divided into 8, 10, 20, or 40 parts, become so many entire animals, each similar to that of which it was at first only a piece" (1743, p. 12). The next confirmation came

from Martin Folkes (1743), President of the society, who presented excellent descriptions of regeneration. The Duke of Richmond (1743) in a letter to the Royal Society described how he had personally witnessed some of Trembley's experiments. Henry Baker (1743) repeated most of Trembley's work and in fact published a book consisting entirely of repetitions of Trembley's experiments before Trembley had published his Mémoires. Finally, George Adams (1746) explained most of Trembley's work in English and stated that he had repeated Trembley's experiments with success.

Thus, Trembley could claim the support of a number of eminent scientists including Réaumur, Buffon, Albinus, Mussenbrock, Allemand, and Folkes. The literary world, however, was not as kind.

Fielding, having seen the Royal Society pamphlet, produced a humorous parody on Trembley's work. Fielding's skit concerns the Chrysipus which he compares to Trembley's polypus. Speaking on the nature of the Chrysipus, its movement, and powers of regeneration, Fielding says, "I have not, after the minutest observation, been able to settle with any degree of certainty, whether this be really an animal or a vegetable, or whether it be not strictly neither, or rather both. For as I have by the help of my microscope discovered some of its parts to resemble those of a lion; I have at other times taken notice of something not unlike the Flower de Luca." (1743,

p. 10).

"But though it hath not, or seems not to have any progressive motion of its own, yet it is easy to communicate a motion to it. Indeed some persons have made them fly all over the town with great velocity" (1743, p. 11 - 12).

"A Chrysipus of the larger kind may be divided into one and twenty substances (whether animal or vegetable we determine not) every substance being at least as large as the original Chrysipus. These may again be subdivided, each of them into twenty-four; and what is very remarkable, every one of these parts is heavier and rather larger than the first Chrysipus. The only difference in this change, is that of the colour; for the first sort are yellow, the second white, and the third resemble the complexion and substance of many human faces" (1743, p. 17).

Speaking on the virtues of the Chrysipus, Fielding writes: ".....A single Chrysipus stuck onto the finger, will make a man talk for a full hour, nay will make him say whatever the person who sticks it on desires....." (1743, p. 29).

Finally, he concluded: "Since I composed the above treatise, I have been informed, that these animals swarm in England all over the country, like the locusts, once in seven years; and like them too, they generally cause much mischief and greatly ruin the country on which they

have swarmed" (1743, p. 31).

Smollett (1751) and Goldsmith (1759, 1762, 1774) were more contemptuous of Trembley's discoveries while Voltaire (1819) even doubted their scientific accuracy, believing the hydra was a plant.

Today, Trembley's work is accepted by most investigators working on the hydra. Many workers have repeated and confirmed Trembley's experiments. Many papers on hydra today refer to or include discussions of Trembley's work, not only for the sake of historical interest. The latter statement bears out a tribute to Trembley paid by Nussbaum, a German biologist, who in 1887 said: "In the year 1744 Trembley's treatise appeared, a masterpiece of precise presentation of carefully and prudently arranged observations, a classical model for a detailed biological investigation that undertakes to give in a single frame a picture of the whole life history of a group of animals. Such a work, accompanied by the skillful illustrations of Lyonet, will forever remain, as regards form and contents, a rich source of instruction for scientific researchers, and will excite joyful admiration through genuine modesty and not easily surpassable clarity of the style" (p. 351).

The fact that the Mémoires remain a source of information to workers on hydra attests to the lasting quality of not only Trembley's work on hydra but his method of study. Trembley's discoveries show that he was a student of processes, not content with purely anatomical descrip-

tions. Trembley was, perhaps, the father of experimental zoology (Garrison, '17; J. Baker, '52). His effect in firmly establishing an experimental approach in medicine has been profound as such an approach is now the basis of most scientific investigation. Karl Ernst von Baer (1864), himself the father of modern embryology, recognized the importance of Trembley's method and observations, for he wrote "....this diligent observation has slowly but substantially influenced physiology, which is the basis of medicine, and thus medicine itself also; and one cannot calculate how many of those present may have profited in days of sickness through such influence, or may yet profit" (p. 109).

Fig. 2 Abraham Trembley performing an experiment before his pupils, Antoine and Jean Sentinck, at Sorgvliet.

Fig. 3 Three green hydra, discovered by Trembley, attached to a plant.

Fig. 4 Regeneration of hydra. Figs. 1 and 2 illustrate two halves of a bisected hydra. Fig. 3 illustrates the closing of the wound in the bottom half. In fig. 4, tentacles are beginning to appear in the bottom half.

Fig. 5 Seven headed "Hydre" produced by repeated splitting of the heads longitudinally.

Fig. 6 Grafting. Fig. 9 One hydra is placed inside of another and fastened with a hog's bristle. Fig. 10 The bottom half of the inside hydra is beginning to push through the body wall of the second. Fig. 11 The graft is permanent at this time.

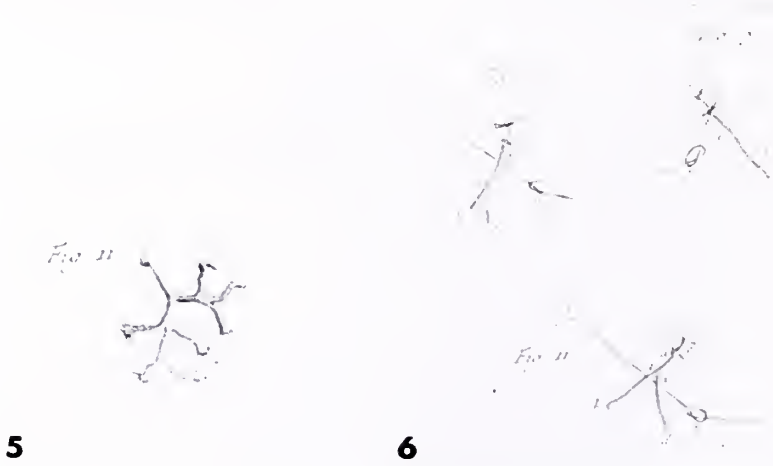
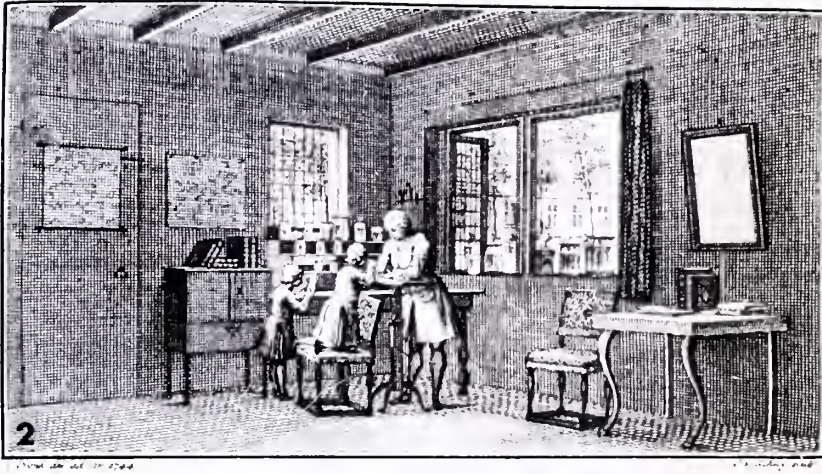


Fig. 7 Budding. The parent hydra, 15 days old, has
19 buds.

Fig. 8



Chapter 2

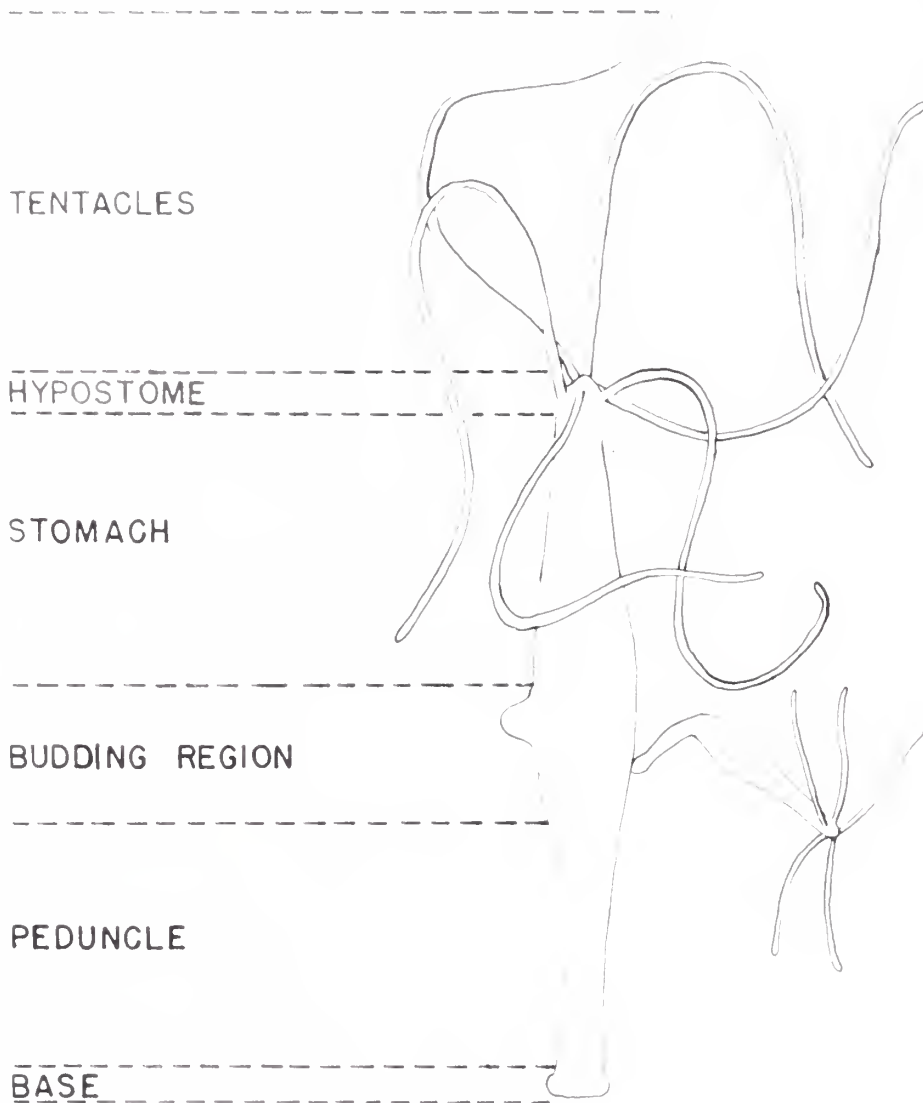
The Biology of Hydra

Since Trembley's time, hundreds of papers have been written on hydra dealing with regeneration, growth, differentiation, reproduction, nematocyst discharge and other biological phenomena. Although a large part of this literature will be discussed in each chapter as it refers to each particular subject, the present chapter reviews some of the more important work concerned with the growth patterns of hydra, regeneration, grafting, induction, polarity, and gradients. The two most recent theories of development which have evolved are presented following a review of the anatomy and histology of the animal.

The hydra is a fresh water coelenterate in the Class Hydrozoa which can be found attached to plants, twigs, and leaves in ponds and streams. It feeds on small crustaceans such as daphnia and cyclops. Reproduction may be asexual by budding or sexual in which eggs and sperm are produced. Under ideal culture conditions in the laboratory hydra may double their number every day by means of budding.

Hydra of different species range in size from 3 mm to 5 cm. A hydra is divided into six regions anatomically, histologically, and physiologically (Text Fig. 1). The animal attaches to the substrate by its base which secretes a sticky mucous substance. An aboral pore is

Fig. 1 Drawing showing the six body regions of hydra. The tentacles are the most distal area and arise in a ring about the mouth which is located in the hypostome. The growth region is located in the distal end of the stomach region below the hypostome. A bud is beginning to form in the budding region and a fully developed bud is about to drop off from the parent. The peduncle is located below the budding region and the animal attaches to the substrate by the base.



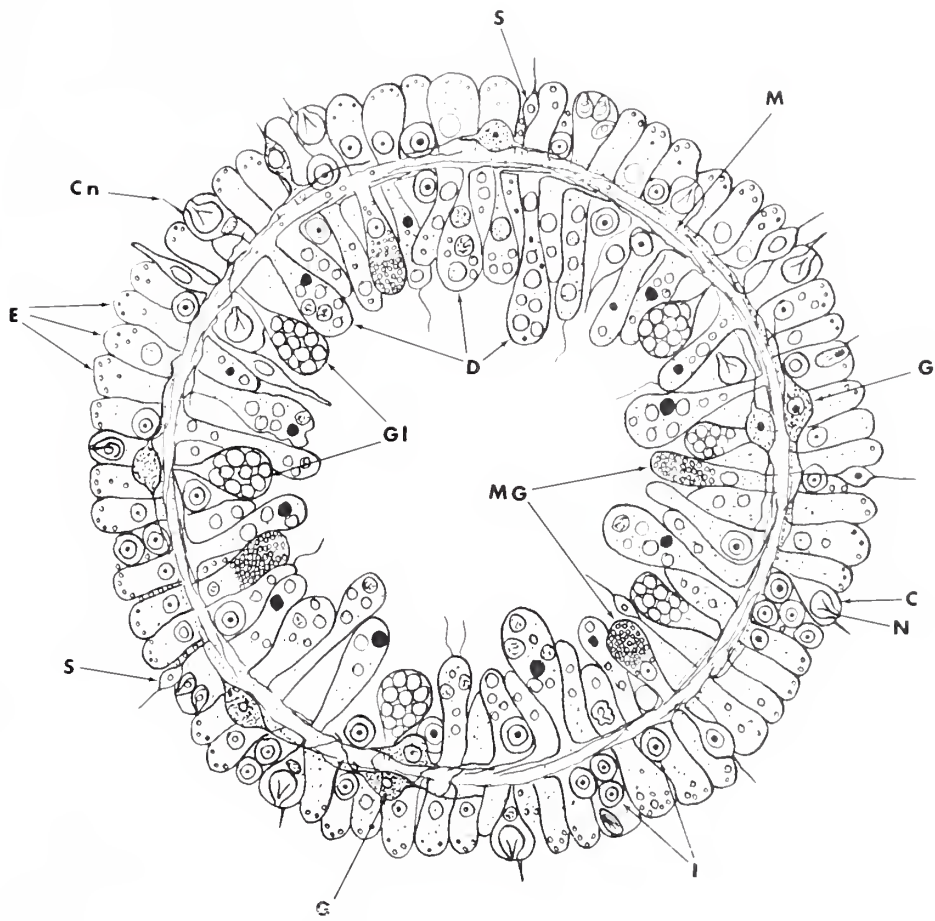
in the base. Proceeding distally, the next region is the stalk or peduncle composed of vacuolated storage cells. The budding region from which new hydranths arise by asexual reproduction lies above the peduncle. Above the budding region lies the stomach where most digestion takes place. The growth region, discussed later, is the distal portion of the stomach region. The most distal region of the body is the hypostome which contains the mouth. The tentacles, four to eight in number, arise from the hypostome in a ring around the mouth and function to capture prey and draw the food to the mouth.

In section (Text Fig. 2; Figs. 3 - 10), hydra is a simple two layered tube of cells surrounding the digestive cavity. The outer layer of epidermal cells is separated from the inner gastrodermis by a supporting lamella or mesoglea. There are seven cell types within the two layers. The chief cell of the epidermis is the epithelio-muscular cell which is cuboidal to columnar in shape and bears a longitudinally drawn out base containing contractile fibers (Figs. 3 - 10). At the base of the hydra these cells are specialized for the secretion of mucus and are termed glandulo-muscular cells (Fig. 10). The interstitial cell is a small undifferentiated cell located chiefly in the epidermis (Fig. 4). They are most numerous in the growth region and absent in the base. This cell is believed to be embryonic in type and capable of differentiating into any other cell type. Cnidoblasts which contain

Abbreviations

B	base	Gm	glandulomuscular cell
BR	budding region	GR	growth region
C	cnidoblast	H	hypostome
Cn	cnidocil	I	interstitial cell
D	digestive cell	M	mesoglea
DC	digestive cavity	MG	mucus gland cell
E	epitheliomuscular cell	N	nematocyst
Ep	epidermis	P	peduncle
G	ganglion cell	S	sensory cell
Ga	gastrodermis	St	stomach region
Gl	gland cell	T	tentacle

Fig. 2 Diagram of a cross section through the stomach region of hydra. Two cell layers separated by the mesoglea surround the hollow digestive cavity. Epitheliomuscular cells, interstitial cells, cnidoblasts, and elements of the nervous system occupy the outer epidermis. The inner gastrodermis is composed chiefly of digestive cells and two types of gland cells.



the nematocysts are most abundant in the epidermis of the tentacles (Fig. 6). The cnidoblast with its enclosed nematocyst is a specialized cell which upon reception of appropriate stimuli discharges a thread which pierces the hydra's prey and injects a poison which immobilizes and kills it. The most abundant cells in the gastrodermis, the nutritive-muscular or digestive cell (Figs. 3 - 8), is columnar with a drawn out base situated circularly at right angles to the processes of epitheliomuscular cells. This cell absorbs food from the digestive cavity and completes the process of digestion intracellularly. Gland cells contain large droplets believed to be digestive enzymes which are released into the digestive cavity during feeding. Mucous cells are most abundant in the hypostome (Fig. 3). Gland cells are mucous cells are absent in the base. The nervous system consists of ganglion cells (Fig. 4), lying above the muscular processes of epitheliomuscular cells, and sensory cells (Fig. 7) situated more superficially in the epidermis. The processes or neurites of these cells form an extensive network in the epidermis.

Growth Patterns

The most important theory concerning the normal growth pattern of hydra was first proposed by Tripp in 1928. He removed the base of a hydra and grafted it beneath the hypostome of the same animal. Over the next several days, the base moved slowly down the body of the animal until it occupied its original position at the foot of the animal. Issajew ('26) had observed a similar

phenomenon in the tentacles. He noted that occasionally a hydra had a fork in one of its tentacles and the fork gradually moved out to the end of the tentacle and finally disappeared. On the basis of these two observations Tripp concluded that the hydra possesses a growth region below the hypostome where cells are constantly dividing and moving proximally and distally. Once a cell reaches the base or tips of the tentacles it atrophies, dies, and is sloughed off.

Brien and Reniers-Deceon ('49) grafted a vitally stained distal portion of a hydra to an unstained proximal portion. Gradually, the stained cells of the distal portion moved proximally reaching the base. Brien, unaware of Tripp's theory, reached an almost identical conclusion that the hydra possesses a sub-hypostomal growth region. Brien went so far as to state that the hydra is immortal due to constant renewal of cells from the growth region replacing those cells at the extremities that are lost because of death and aging.

Theories of Development

Investigators have performed innumerable grafting and regeneration experiments involving different parts of hydra (Wetzel, 1895; Feebles, 1897, 1900; Rand, 1899a, 1899b, '11; Parke, 1900; King, '01; Hefferan, '02; Brown, '09; Koelitz, '11; Burt, '25; Issajew, '26; Rand et al, '26; Tripp, '28; Weimer, '28, '32, '34; Mutz, '30; Papen-

fuss, '34; Yao, '45a, '45b). These experiments resulted in two important findings. First, the distal portion of a regenerating hydra always developed a head while the posterior surface developed a base. In other words, portions of hydra rigorously maintain their polarity. Secondly, the hypostome of hydra is the dominant center in the animal. This finding was demonstrated most conclusively by Browne in 1909 working with T. H. Morgan. She found that grafting a piece of hypostome to the body of another hydra resulted in the outgrowth of a new hydranth at this point. Buds and tissue that had regenerated for at least 10 hours produced similar results. In order to determine the origin of the tissue comprising the new hydranth, she grafted the hypostome of an originally green hydra which had been cleared of its symbiotic algae (Whitney, '07, '08) on to a green hydra. The result of this graft was the outgrowth of green tissue carrying the grafted portion of white tissue at its end. This experiment demonstrated that the material of the body wall of the stalk and not the hydranth of the graft forms the new hydranth, a clear example of induction.

The first theory to account for the maintenance of polarity and inductive power of the hypostome was the gradient theory of Child ('42). Several workers previously had presented evidence for axial gradients in hydra. There was a proximal-distal increase in the number of

tentacles formed on regenerating hydra, i.e., the nearer the cut was made to the hypostome, the more tentacles formed on the proximal portion (Peebles, 1897; Burt, '25; Weimer, '28). There were distal-proximal gradations in rate of disintegration of hydra in toxic solutions and in rate of potassium permanganate reduction (Child, '19). Distal-proximal gradations have been revealed also by photolysis (Hinrichs, '24) and by methylene blue reduction (Child, '34). These results indicated the distal portion of the hydra, the hypostome, was a locus of "high activity". Secondary regions of high activity were also present in the base (Child and Hyman, '19) and in the budding region (Weimer, '28).

The theory proposed by Child which he applied to the entire animal kingdom was that metabolism is the effective factor of development (Child, '41). Accordingly, metabolism in a spatial pattern is the primary factor in producing regional differences in rate of development and in determining form of the organism. Thus, in the case of hydra, the hypostome was a region of high metabolic activity dominant over the rest of the animal. Implants of hypostome into other animals acted as inductors determining new apical regions and polarities from host tissue by virtue of the hypostome's dominant metabolic activity. Physiological and external factors which are effective in altering or determining developmental pattern act by

influencing the metabolism of living protoplasm.

Certain objections, however, have been raised against Child's theory. This theory of metabolic gradients, plausible in animals such as planaria with a single apico-basal gradient, is greatly complicated by animals such as hydra with more than one region of high activity. The hydra presumably has three regions of high metabolic activity: the hypostome, the budding region, and the base. Each is markedly different in structure and function. The theory of metabolic gradients does not explain these differences in regions which all have high activity. Secondly, if one were to remove a section of hydra extending from the middle of the budding region to the middle of the stomach region, one would expect tentacles to develop from the proximal (bottom) end of the segment which has higher activity. In actuality, the reverse happens since tentacles develop from the distal end of any portion of hydra.

Most recently, Burnett has developed a concept of growth stimulators and inhibitors to explain the maintenance of form in hydra. He grafted the growth region of one species of hydra to the peduncle of another species. In a few days, the distal portion of the first species began to form new tentacles and hypostome and in addition, tentacles began to grow from the proximal portion of the second species. It was suggested that a growth

stimulatory principle diffused from the tissues of the first species into the second and stimulated the formation of a new hypostome and growth region.

Burnett and Lesh (personal communication) have attempted to concentrate this factor by placing hypostomes on agar blocks into which the growth substance presumably diffuses from the tissues. When applied to the body of another animal the agar blocks are capable of eliciting tentacle formation.

In order to explain the dominance of the hypostome over other regions, Burnett postulates that an inhibitive substance is produced in the growth region which inhibits cellular division proximally. The effect of the growth inhibitory factor diminishes at the level of the budding region where a secondary region of cell division is established. Growth inhibitory substance is produced again in the budding region which diffuses proximally inhibiting the peduncle and base. This hypothesis is based on the observation that the grafting of a peduncle between the growth and gastric regions results in tentacle formation at the distal portion of the gastric region. Presumably, the cells of the peduncle impede the passage of growth inhibitor proximally from the growth region. Thus, to summarize Burnett's views, the form of any body region of the hydra is controlled by a balance between a growth stimulatory substance produced in the hypostome and a

growth inhibitory substance produced in the growth region. The relationship of the present studies to these theories is discussed in subsequent chapters.

In summary, the hydra is structurally and organizationally one of the simplest metazoans. And yet, as will become clearer in the following chapters, this simple organism exhibits all the cellular functional specializations present in higher invertebrate and vertebrate forms. The fact that these processes, ranging from cell permeability to neurosecretion, can be studied in an evolutionally simple form and in relation to the entire organism is perhaps responsible for the widespread and increasing use of this animal as a tool in experimental biology.

Explanation of Figures

See p. 53 for abbreviations

Fig. 3 Longitudinal section through the hypostome. The two cell layers surround the digestive cavity. Foamy vacuolated gland cells occupy the gastrodermis near the mouth area. The base of a tentacle protrudes from the hypostome. 300X

Fig. 4 Growth region. Note the numerous small interstitial cells in the epidermis. Two ganglion cells are present in the epidermis near the mesoglea. 1200X

Fig. 5 Transverse section through a hydra in the stomach region. Note the basic organization of the animal consisting of two cell layers surrounding a hollow digestive cavity. 240X

Fig. 6 High magnification of the stomach region. Two nematocysts are present in the epidermis. The digestive cells are large and contain food and fat vacuoles. 1200X

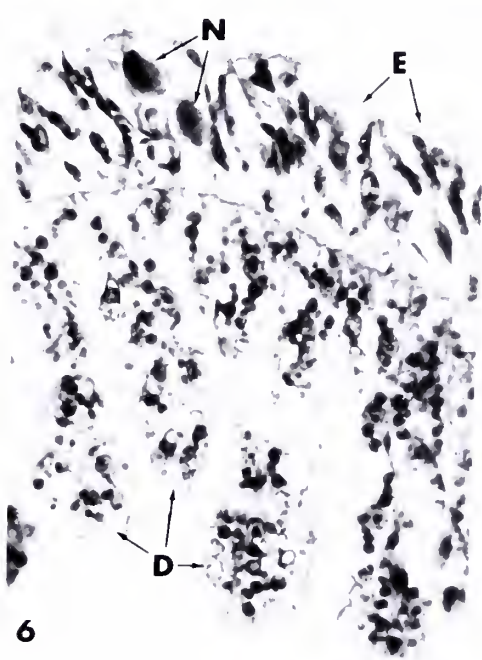
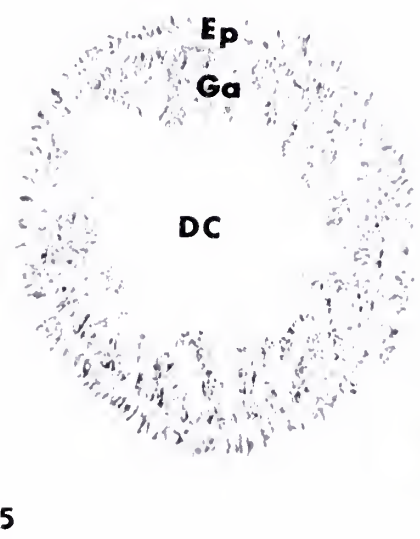
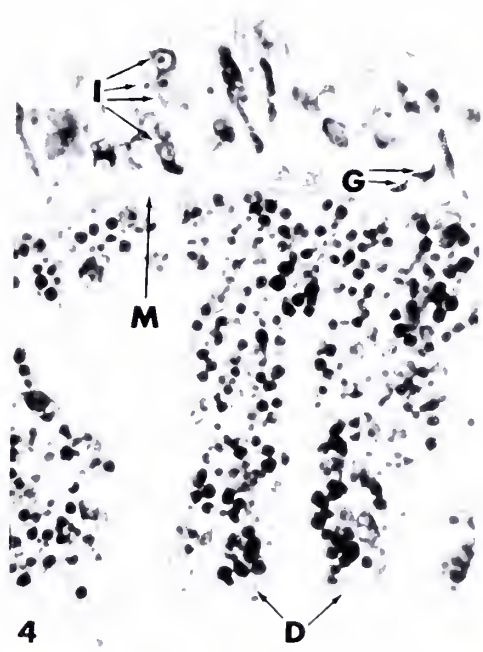
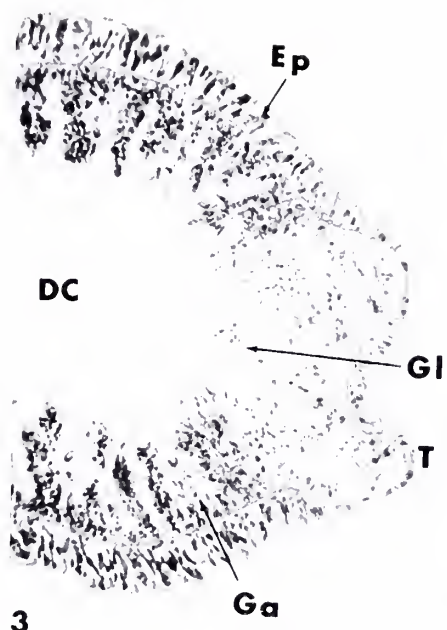
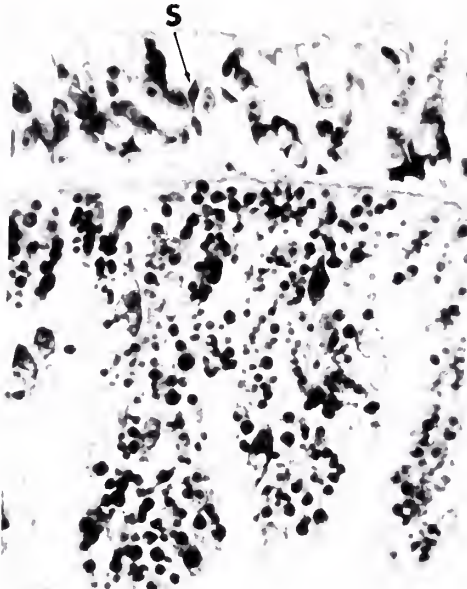


Fig. 7 Section through the stomach region. A sensory cell is present in the epidermis. 1200X

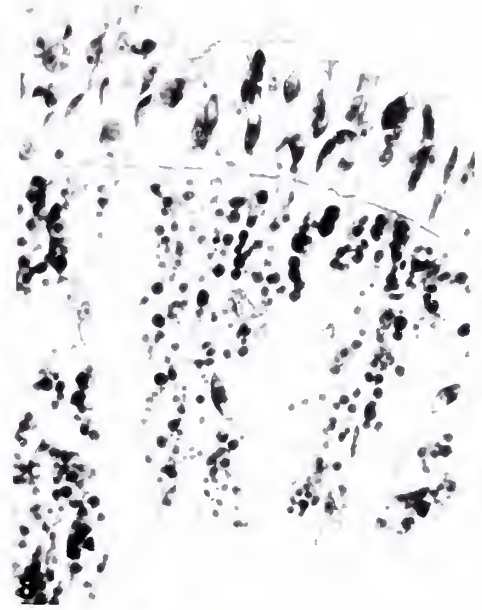
Fig. 8 Budding region. Small differentiating cells are present in the epidermis. Note that the digestive cells are not as large as those in the stomach region. 1200X

Fig. 9 Peduncle. The epitheliomuscular cells are cuboidal in this region. The digestive cells contain large intracellular spaces. 750X

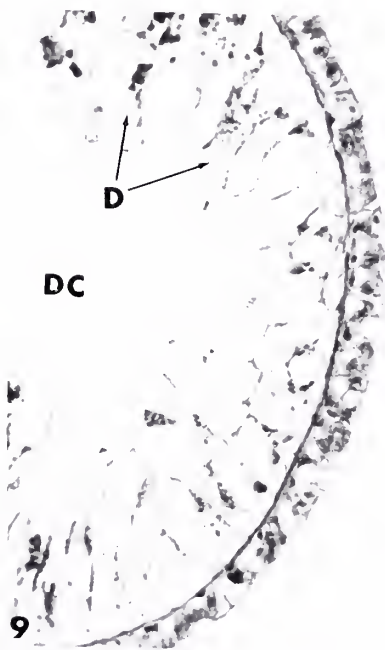
Fig. 10 Base. The epithelial cells are columnar and filled with tiny mucus granules. The digestive cells are small and atrophic. Fragments of cells (arrow) occupy the digestive cavity and are lost through the basal pore. 750X



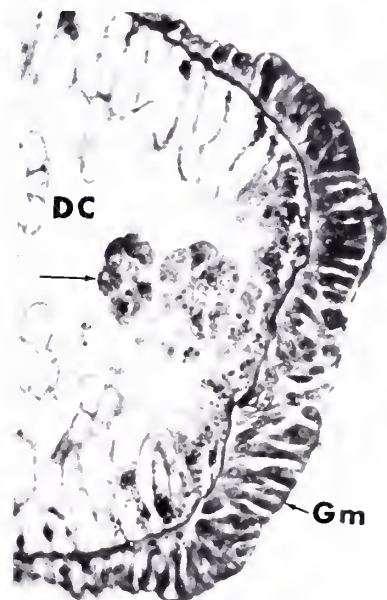
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Chapter 3

Enzyme Histochemistry of Hydra

These studies were undertaken to localize enzymatic activities by use of histochemical techniques prior to the investigation of the role of enzymes in processes of differentiation and permeability in the hydra. As this study proceeded, the exceptional interest that the hydra holds for biological investigation was rediscovered on an enzyme histochemistry basis. This coelenterate, which has one of the most simple and fundamental structures of all multicellular animals, showed such extremely interesting intracellular as well as regional localizations of enzyme activity that the study was extended to include thirteen enzyme histochemistry methods. Of particular interest were the results that focused on problems concerned with the relationship of enzyme activity to nematocyst discharge and to questions concerned with the existence of a primitive nervous system. Although histochemical investigations on hydra have been carried out by Yoder ('26), Semal-Van Gansen ('54), Burnett, ('59), and Cowden ('60), no comprehensive study of enzyme histochemistry has been performed. The histochemical localization of enzyme activity in the epidermis, gastrodermis, and the nematocysts of hydra were studied. The enzyme localizations investigated were mainly several oxidative systems, some of the carboxylic acid esterases,

and some of the phosphatases.

Materials and Methods

Hydra littoralis and Hydra pseudoligactis, maintained successfully in culture for over a year by the method of Loomis and Lenhoff ('56) modified by Burnett ('59), were used in these experiments. Formalin-fixed, paraffin-embedded sections were not optimal material for histochemical tests because some enzyme activities were seriously inhibited by the routine fixation and embedding. Intact hydra, either unfixed or fixed for 10 minutes in ice cold 10% formalin containing 1% CaCl_2 , and then immersed in the incubating media gave best results. Although the intact unfixed or fixed cells of the hydra might not admit some of the histochemical substrates and reagents, this was not found to be the case for any of the histochemical tests used. The hydra cells were permeable to substrates and reagents and reacted with excellent cytological localization of enzymatic activity in a few minutes. After incubation, the hydra were either fixed briefly in the cold formalin and mounted in glycerine jelly or mounted in glycerine jelly without further fixation. The epidermis of the whole mounts were examined by means of ordinary light microscopy. Although the cells of the gastrodermis also reacted in these specimens, microscopic observations were restricted to the epidermis by selection of the plane of focus.

The results of the histochemical tests on the gastrodermis were observed directly in hydra that were turned inside out by a simple procedure. Small pieces of silver or platinum wire about 5 mm long were inserted upright into a paraffin layer on the bottom of a Syracuse watch glass filled with water. With the aid of a dissecting microscope (24X) a hydra was impaled, base first, over the upright wire using a watchmaker's forceps. The hydra, thus standing upright with the wire occupying the gastric cavity, was incised by making a longitudinal split down one side with a scalpel. The animal was then easily everted with forceps. These hydra maintained their everted position throughout incubation so that they could be mounted and observed with the gastrodermis facing out.

The enzymatic activities observed in the whole mounts were localized in sectioned hydra. Enzymatic activities comparable to those observed in the intact animal were noted in sectioned material fixed briefly in formalin as described above and embedded in polyvinyl alcohol (PVA) (Feder, '59). This embedding medium preserved enzymatic activity better than paraffin because the tissues were not exposed to high temperatures or to the effects of the dehydrating and clearing agents. Five μ sections were mounted on slides and incubated in coplin jars. The thin sections revealed enzymatic

localizations similar to those of the whole mounts with the exception of succinic dehydrogenase activity which was seriously inhibited. With this exception the observations described in a following section hold for all methods of preparation.

Histochemical Techniques

Histochemical tests demonstrated the activity of a variety of hydrolytic and oxidative enzymes in the hydra. The methods were those routinely employed and slightly modified to fit the differences between the use of hydra and mammalian tissues and the differences between incubating sectioned material and intact small animals. Because most of these methods are in common use and have already been reviewed (Deane, Barrnett, and Seligman, '60; Pearse, '60), enumeration of the incubation media is not required. The slight modifications referred to above included either small changes in pH of the incubating medium, or more usually, use of more than the suggested amount of capture reagent.

A. The Oxidative Enzymes

Succinic dehydrogenase activity was demonstrated by the method of Nachlas et al ('57) using the ditetrazolium salt, Nitro BT, as an electron acceptor and sodium succinate as substrate. In some experiments endogenous dehydrogenase activity (that obtained without the use of exogenous substrate) was destroyed by placing the hydra

in saline at 0°C for 15 minutes before incubation. The usual procedures of freezing and thawing to destroy endogenous activity could not be used in these experiments since they disrupted the hydra as well as destroyed the endogenous activity. It should be noted, however, that the sites of localization of succinic dehydrogenase activity were the same as those of the combined activities of succinic dehydrogenase and endogenous dehydrogenase.

Reduced coenzymes, DPNH and TPNH, were used as substrates with Nitro BT to determine the sites of the oxidative enzyme systems that require pyridine-nucleotide coenzymes, diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN). This was done instead of using specific substrates such as lactic acid or glucose-6-phosphate, since electrons are transferred from the specific substrates to the coenzymes and from the reduced coenzymes to a diaphorase. This method therefore reveals DPNH or TPNH diaphorase activity.

The following control procedures were carried out: omission of substrate from the incubating medium to determine if the ditetrazole was being reduced non-enzymatically or by endogenous dehydrogenase activity; pre-incubation in N-ethyl maleimide (0.04M) to block sulfhydryl groups; addition of malonate (0.02M) to inhibit succinic dehydrogenase; and heat inactivation by placing the hydra in boiling water for 1 minute before incubation.

B. The Hydrolytic Enzymes

1. The Carboxylic Acid Esterases

Carboxylic acid esterase activity was demonstrated by an azo dye method using α -naphthyl acetate as substrate (Gomori, '50) and different diazonium salts, especially Diazo Blue B, as reagents. In addition, the combination of thiolacetic acid and lead nitrate in cacodylate buffer (Barrnett and Palade, '59; Barrnett, '61) was used; this method was recently appraised by Wachstein, Meisel, and Falcon ('61). Because both substrates may be hydrolyzed by non-specific esterases or aliesterases, lipases, and cholinesterases, the reactivity of various sites in the hydra was compared with the reactions of the liver, pancreas, and motor end plates of skeletal muscle of the rat. For these experiments different inhibitors were used in addition to the regular incubation to distinguish between some of the different carboxylic acid esterases. Those sites in the hydra that reacted similarly to pancreatic zymogen granules and were not inhibited by sodium taurocholate ($1 \times 10^{-3} \text{M}$) were considered similar to lipase; those that reacted similarly to the enzyme in rat liver and were inhibited by taurocholate but not by eserine salicylate ($1 \times 10^{-5} \text{M}$) were considered non-specific esterase or aliesterase; and those that reacted similarly to the motor end plates and were completely inhibited by eserine, 1:5-bis-(p-trimethylammoniumphenyl) pentan-3-one diiodide

(62C47) ($10^{-6}M$), and N-p-chlorophenyl-N-methyl carbamate of m-hydroxyphenyltrimethylammonium bromide (NU 1250) ($1 \times 10^{-5}M$) were considered a cholinesterase. For these experiments, the hydra were incubated briefly in 13% sucrose containing the inhibitor followed by incubation in the regular medium also containing the inhibitor. In other experiments the inhibitor was merely added to the medium. Thiolacetic acid may, in addition, be split by other enzyme systems such as acyl transferases (Barrnett and Palade, '59) but this possibility was not investigated with the hydra.

2. The Phosphatases

Alkaline phosphatase activity at pH 9.1 was determined by two methods: the metal salt method of Gomori ('52) utilizing sodium glycerophosphate as substrate and the azo dye technique (Gomori, '51) employing a-naphthyl phosphate as the substrate with Diazo Blue B as reagent. Control reactions were obtained by including the inhibitor cysteine ($5 \times 10^{-3}M$) in the incubating medium.

Acid phosphatase activity at different pH's (5.1 to 6.4) was investigated by the metal salt (lead) method of Gomori ('50a), recently reviewed by Holt ('59), with sodium glycerophosphate as substrate. An azo dye technique, employing a-naphthyl phosphate and Garnet GBC as the diazotate (Burstone, '58), was used for comparative purposes. Experiments were performed with sodium fluoride

($1 \times 10^{-2} \text{M}$) as inhibitor.

The activity of several other specific phosphatases were investigated and compared with those of alkaline and acid phosphatases. Naming these enzyme activities after the substrate used is a matter of convenience and does not imply the specificity of enzyme for that substrate or the similarity to mammalian enzymes that have the same name. There was considerable overlap of the sites of activity of the enzymes splitting the special phosphatase-containing substrates and marked differences between these and alkaline and acid phosphatase activities.

5-nucleotidase activity with adenosine-5'-phosphate as substrate was investigated by the method of Wachstein ('55) at pH 7.2. Nickel salts (10^{-2}M) which do not inhibit alkaline phosphatase were used in the inhibition experiments.

Adenosinetriphosphatase activity was demonstrated by the method of Wachstein and Meisel ('56) using adenosinetriphosphate as substrate with lead nitrate as capture reagent. Either phlorizin (10^{-2}M) or N-ethyl maleimide (10^{-2}M) were added to the incubating medium as inhibitors.

Enzyme activity that hydrolyzed glucose-6-phosphate was demonstrated by the method of Wachstein and Meisel ('56). Inhibition studies were performed on material pretreated with acetate buffer at pH 5 (Beaufay and deDuve, ('54).

3. Peptidases

The activity of the enzyme(s) which hydrolyze L-leucyl-B-naphthylamide (Green et al, '55; Folk and Burstone, '55) were investigated by the method of Burstone and Folk ('56). Enzyme activity was inhibited by Cu^{++} (10^{-2}M). The substrate is hydrolyzed by several amino peptidases (Patterson, Keppel, and Hsiao, '61), one of which may be leucine aminopeptidase, and therefore the method cannot be considered specific.

In addition, a number of proteolytic enzymes may split carboxylic acid esters. Both substrates used to localize esterases, especially thiolacetic acid, are split by these enzymes, including presumably cathepsin C (Hess and Pearse, '58) which is resistant to heat and diethyl p-nitrophenylphosphate (E600). This activity is localized to lysosome-like structures in rat tissues (Wachstein et al, '61) and is clearly distinguishable on a morphological basis from the more diffuse cytoplasmic esterase reaction.

Observations

Oxidative enzymes

Succinic dehydrogenase activity appeared evenly distributed throughout all regions of the hydra. The activity appeared in all cell types of both epidermis and gastrodermis and was localized to small granules dispersed throughout the cytoplasm (Fig. 3). No activity occurred

within the nemtatocysts although the cytoplasm of the cnidoblasts contained many reactive granules (Fig. 4). When the substrate, succinate, was omitted from the incubating medium, no blue deposits of diformazan appeared in the hydra that had been previously washed in cold saline. For that matter, very little endogenous dehydrogenase activity occurred without substrate even in unwashed hydra. Heat (100°C for 1 minute), N-ethyl maleimide, and malonate completely inhibited enzyme activity of all cells.

DPHN diaphorase activity was intense in the cells of the tentacles, hypostome, and base (Figs. 5 and 6). The reaction in the cells of the stomach, budding region, and peduncle was at best sparse (Fig. 6). However, the localization of activity was the same for all cell types of both layers in all regions; the differences being largely due to the amount of activity. This reaction appeared for the most part confined to tiny granules which were smaller than those which showed succinic dehydrogenase activity. These granules were scattered throughout the cytoplasm but many of them also appeared located at the cell surfaces (Fig. 7). In the digestive cells, these granules were also attached to the membranes surrounding the numerous vacuoles. In all cells only a few of the larger type granules, which showed succinic dehydrogenase activity, were active for DPNH diaphorase.

No DPNH diaphorase activity occurred in the hydra washed in cold saline and incubated in the absence of substrate. In addition, N-ethyl maleimide completely blocked the reaction. Brief fixation in cold formalin only slightly inhibited this enzyme system in contrast to succinic dehydrogenase which was almost completely inhibited.

The localization of the activity of TPNH diaphorase activity was similar to that of DPNH diaphorase. Activity appeared in the cells of all regions, mainly in the form of tiny granules within the cytoplasm and associated with cell membranes and the membranes of vacuoles (Fig. 8). As with DPNH diaphorase, an occasional cell of unknown type showed so much activity so as to be confluenty stained (Fig. 8). The cells of the stomach, budding region and peduncle showed less activity than the other areas. No activity appeared within the nematocyst but many tiny granules occurred in the cytoplasm of the cnidoblast as well as in relation to the cell surface (Fig. 9).

Hydrolytic enzymes

Non-specific esterase (taurocholate sensitive and eserine insensitive) activity was distributed throughout all regions of the hydra. The cell surfaces of all epidermal cell types were active (Figs. 10 and 11). In most cells, the plasma membrane showed activity but even more conspicuous were numerous large or small reactive vacuoles

that were related to the cell surface (Fig. 11). Most of these stained vacuoles appeared to be intercellular, but a few were clearly localized in the cytoplasm of the epidermal cells. The surface membranes of the cnidoblasts and the cnidocil were reactive but no activity appeared within the nematocyst itself. The digestive cells of the gastrodermis like the epidermal cells showed activity at cell surfaces, in intracellular vacuoles of several sizes, and in intercellular vacuoles. In the gland cells, esterase activity was intense in the cytoplasm immediately surrounding the large spherical granules which showed weak activity.

There was no enzyme in the hydra that corresponded to rat pancreatic lipase, since no epidermal or gastrodermal activity could be demonstrated in any cell type in the presence of sodium taurocholate.

Cholinesterase activity was present in all regions of the hydra but predominantly in the cells of the hypostome and base. Many small reactive granules appeared located at the plasma membrane of the epithelio-muscular cells of all areas (Fig. 12). Large bodies, presumably ganglion cells, laden with dense accumulations of final product of the enzyme reaction, were present between the bases of the epithelio-muscular cells (Figs. 13 and 14). These were most numerous in the hypostome and base (Fig. 13). A few similarly reactive bodies, presumably sensory

cells, were located between the apical portions of the epithelio-muscular cells of the epidermis (Fig. 15). Similar localizations of cholinesterase activity to that found in the epidermis occurred in the gastrodermis and consisted of small stained granules at the surface of digestive cells and reactive bodies located between them. A small extracellular cluster of intensely reactive granules was usually found near both digestive and epithelio-muscular cells.

Nematocysts of all types, but only those in the budding region, stomach, and hypostome, showed cholinesterase activity within the capsule (Fig. 16). In the peduncle and tentacles, there was no activity within the nematocyst (Fig. 15). However, grape-like clusters of small round reactive bodies appeared attached at one point to the surface of the cnidoblasts in the peduncle and tentacles (Figs. 17 and 18). Many cnidoblasts of all regions possessed an accumulation of reactive granules within their cytoplasm (Fig. 19).

Linear, extracellular, fibrillar structures in the epidermis and gastrodermis of all body regions also showed enzymatic activity. These fibers formed a continuous network connecting the dense granular bodies at the bases of the epidermal and digestive cells (Fig. 14). In addition, they were often seen connecting the dense reactive bodies (ganglion cells) and the grape-like reactive clusters of

granules on the cnidoblasts (Fig. 18).

Activity of the above sites was usually inhibited by eserine, 62047, and NU-1250. Some structures like the ganglion cells and the grape-like clusters of granules showed a little residual activity. Since these were the most reactive structures, by far, their slight staining was probably due to incomplete inhibition. Enzyme activities within nematocyst capsules were only partly inhibited by eserine but completely inhibited by 62047 and NU-1250. Enzymatic activity of linear fibrils was completely inhibited by all reagents.

Non-specific alkaline phosphatase activity occurred in the epidermis of all regions of the hydra, but more intensely in the tentacles, peduncle, and base. In the epidermis, activity of the enzyme-like non-specific esterase, appeared at cell membranes, in the membranes of intracellular and extracellular vacuoles, and in the contents of cytoplasmic droplets (Fig. 20). In the digestive cells which were active in all regions, the enzyme activity was localized in cell membranes and in vacuoles of different sizes (Fig. 21). Hypostomal mucous cells and gland cells showed more intense activity than the other cell types. Cnidoblasts of all regions were active, the enzyme being localized on the surface membrane within and on the capsule of the nematocyst (Fig. 21) and in the cnidocil.

Acid phosphatase activity was seen in all regions of the epidermis but was less intense in the cells of the hypostome, stomach, and budding region. Activity occurred in cytoplasmic vesicles which were numerous in the cells of the tentacles, peduncle, and base. These small vesicles frequently bore a close relationship to the cell surface and large enzymatically active vesicles also occurred deeper in the cytoplasm (Fig. 23). Similar small and large stained vesicles occurred within the cytoplasm of the cnidoblasts of all regions. In addition, the cnidocils and capsules of the nematocysts were intensely reactive but only in the tentacles and peduncle (Figs. 24 and 25). The digestive cells of the gastrodermis contained active cytoplasmic vesicles of various sizes, even large reactive vacuoles (Fig. 26). Gland cells contained intensely reactive large vacuoles while the hypostomal mucous cells possessed mainly smaller active vesicles (Fig. 27).

A problem arose when specific substrates were used to localize the activity of specific phosphatase enzymes because the sites of activity either showed considerable overlap or were often identical when adenosine-5'-phosphate, adenosinetriphosphate, and glucose-6-phosphate were used as substrate. It should be noted, however, that the sites of activity of the enzymes hydrolyzing these substrates were quite different from that obtained

with alkaline or acid phosphatase. Inhibition studies gave additional information concerning this dilemma since the activity demonstrable when adenosine-5'-phosphate was used as substrate was inhibited by Ni^{++} that destroys 5'-nucleotidase activity. When adenosinetriphosphate was used as substrate, the reaction was obliterated by phlorizin or N-ethyl maleimide. But, when glucose-6-phosphate was used as substrate the reaction was not disturbed by treatment with buffer at pH 5.

The activity of the phosphatases hydrolyzing the specific substrates was localized intensely in the tentacles, peduncle, and base but a less intense reaction also occurred in the other areas. With each of the special substrates, a circular band of intensely reactive cells was situated between the tentacles and the hypostome (Fig. 28) and between the peduncle and the budding region (Fig. 29). Activity in epidermal cells was confined to cell membranes and to tiny granules or vesicles which were attached to the cell membranes (Figs. 30 and 31). The activity in epidermal cells was more conspicuous when adenosine-5'-phosphate was used as substrate (Fig. 31) and comparison of Figures 29, 30, and 31 indicates the differences in localization which were sometimes encountered when the three substrates were used. The cnidocils, as well as the capsules of all types of nematocysts, present in only the tentacles and peduncle showed

activity with all substrates (Figs. 28 and 32). The surface membrane of the cnidoblasts containing the nematocysts was also positive. Digestive cells showed activity in cell membranes, small granules attached to cell membranes, and in vacuoles of all sizes within the cells (Figs. 33 and 34). When glucose-6-phosphate was used as substrate, the contents of small basilar droplets were active, a localization seen only occasionally when the other phosphates were used as substrates. Gland cells showed activity either on the surface or in the contents of large vacuoles and hypostomal mucous cells were filled with tiny reactive granules.

Peptidases

Aminopeptidase activity was evenly distributed throughout the cells of the epidermis. The cell membranes of the epidermal cells were active as well as associated granules and vacuoles (Figs. 35 and 36). Activity also appeared in the surface membranes of the cnidoblast and on the cnidocil (Fig. 37), but there was no activity within the nematocyst. All cells in the gastrodermis showed intense activity localized at the cell membranes as well as vesicles which were often associated with the cell surface (Fig. 38). The latter gave the appearance of pinocytotic vesicles being pinched off from the cell membrane (Figs. 36 and 38). Gland cells showed intense activity within their cytoplasmic

vacuoles. As indicated previously, thiolacetic acid also appears to be hydrolyzed by cathepsin C. Therefore, it was not surprising to find that the round cytoplasmic bodies which were identical to the acid phosphatase positive bodies also reacted intensely with the thiolacetic acid method.

Discussion

These experiments demonstrate a remarkable regional as well as intracellular localization of enzyme activity in hydra. The results appear to be significant in relation to earlier research on growth and metabolic gradients and on the initiation and mechanism of nematocyst discharge. In fact, this avenue of histochemical investigation reveals sufficient promise as an approach that in the hydra it could provide a solution to some of the of the previously perplexing problems in these areas.

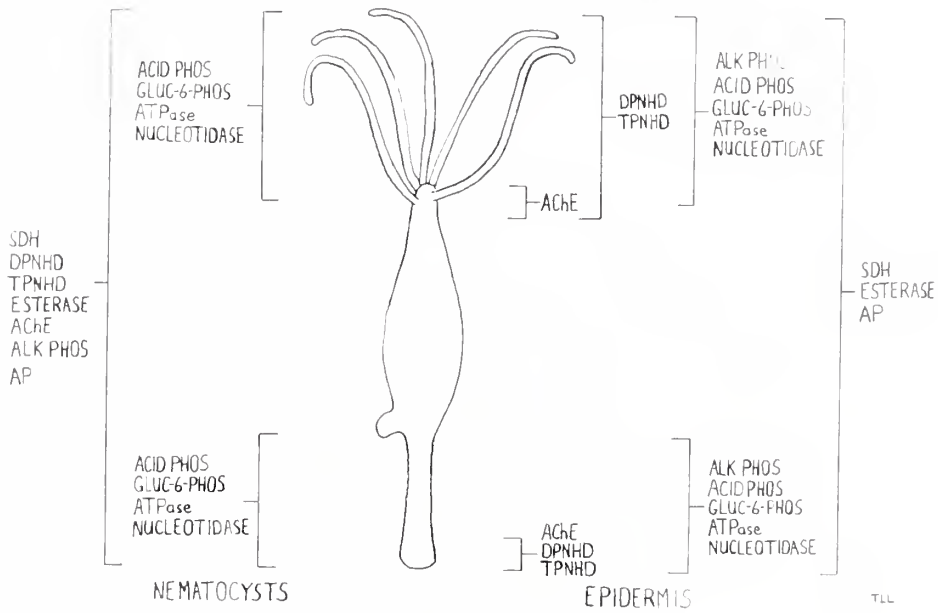
This work on enzyme distributions indicates that as cells undergo morphological and functional specializations they undergo enzymatic specialization. In this regard, it should be recalled that the hydra is an "immortal animal" (Brien and Reniers-Decoen, '49) because of the constant cell proliferation and renewal in the growth region. This finding ^{was} /first enunciated by Tripp ('28) and more recently studied by Brien and Reniers-Decoen ('49), Burnett ('59), and Burnett and Garofalo ('60) who

noted and described a growth region below the hypostome in which cells are constantly proliferating and moving distally up the tentacles and proximally toward the base. As cells migrate proximally and distally from the growth region they undergo morphological and functional changes so that specific body regions in hydra contain morphologically distinct cell types. In addition, differences in metabolic activity of different regions has been hypothesized (Child and Hyman, '19; Hinrichs, '24; Weimer, '28, '32; Child, '47). More recently Burnett ('59) has shown that morphological and histochemical differences exist in different cell types during periods of feeding and starvation.

The conclusions of these previous workers is re-emphasized by the histochemical distribution of enzymatic activities. The region between the hypostome and peduncle (including the growth region, stomach, and budding region) is unspecialized enzymatically (Text Figure 1, right). These regions contain the activities of succinic dehydrogenase, non-specific esterase, and aminopeptidase which, in addition, are found in all other areas and therefore appear basic to the entire animal. Only when the epidermal cells have migrated distally to the hypostome and tentacles and proximally to the peduncle and base do they acquire enzymatic specialization. Thus, as the cells differentiate, they acquire the activities of

Text fig. 1 On the right hand side, the extent of the most intense localizations of the enzyme activity in the epidermis is indicated. Similarly, on the left hand side, the regional distribution of enzyme activity in the nematocysts is shown.

REGIONAL LOCALIZATIONS OF ENZYMES



mainly the diaphorases and the phosphatases. These findings, therefore, indirectly support the notion of the presence of a growth region below the hypostome and directly indicate that enzymatic development proceeds along with morphological development.

A parallel situation was found concerning the development of nematocysts and the presence of enzymes. It should be recalled that Brien and Reniers-Decoen ('49), Semal-Van Gansen ('51), and Burnett and Lentz ('60) suggested that nematocysts are formed in the body region and the cnidoblasts that contain them move proximally and distally. In addition, nematocysts of the body are incapable of firing while those in the tentacles and peduncle are functionally fully developed. However, many nematocysts of the stomach and hypostome are morphologically identical with those of the tentacles, but they are not enzymatically identical.

The histochemical localizations of enzyme activities in cnidoblasts and nematocysts are summarized in Text Figure 1 (left). When the cnidoblasts migrate to the tentacles and peduncle, their nematocysts develop the full complement of enzymes and only then are these structures fully mature. The nematocysts found in other regions (hypostome, stomach, and budding region) do not contain the activity of the special phosphatases present in the active nematocysts of the tentacles and peduncle.

However, the attainment of these enzymatic activities was not a gradual process. Instead, there appeared to be definite lines of demarcation at the base of the tentacles and between the peduncle and budding region. On one side of the line the nematocysts were enzymatically active; on the other, inactive. If nematocysts gradually matured chemically with respect to time only, one would expect a gradual increase in enzyme activity from the stomach to the tentacles or peduncle. However, the sharp regional differences suggest that the nematocyst becomes enzymatically mature suddenly, possibly due to intracellular or to local influences. This notion is in agreement with the results of Burnett ('60) who found differences in the pH signature staining of nematocysts in different areas and as a result of transplants. Accordingly, he felt that the nematocysts in the stomach region were held in chemical abeyance but that this inhibition was released (along with an increase in acidity) once the nematocyst was brought under the influence of the tentacles or peduncle.

The present histochemical results also bear suggestive information concerning the control of nematocyst discharge. Two views exist to explain the mechanism controlling the discharge of nematocysts. One theory assumes that the discharge of nematocysts is dependent on nervous control (Chun, 1881; Lendenfeld, 1887; Murbach,

1893; Grenacher, 1895; Glaser and Sparrow, '09). The other hypothesis suggests that the nematocyst is an independent effector which is dependent on local mechanical and chemical stimulation for eversion (Iwanzoff, 1896; Parker and Van Alstyne, '32; Pantin and Pantin, '42; Pantin, '42a, b; Ewer, '47; Picken, '53; Robson, '53; Jones, '47; Burnett, Lentz, and Warren, '60).

Part of the present observations support the latter view. The cnidocil and/or the capsule of the mature nematocysts contains enzymes that can hydrolyze seven of the substrates used in the present experiments (Text Fig. 2). This strategic localization suggests that the enzymes may be directly involved in the triggering of the nematocysts. Upon reception of an appropriate chemical stimulation, chemical reactions associated with enzyme activity could set the stage for the discharge of the nematocyst. Moreover, nematocysts of the body region, not having developed the strategic enzymes, are unable to respond to a chemical stimulus which might serve as a substrate for the enzyme and therefore as the effector substance. In the foregoing, the triggering mechanism has been purposely separated from the discharge mechanism but it would seem highly probable that both were enzymatically controlled. The frequent association of adenosinetriphosphatase activity with different forms of motility may be especially pertinent in the discharge

Text fig. 2 The cytological localizations of the enzyme activity in an epitheliomuscular cell, a digestive cell, and a cnidoblast are shown in this diagram.

ENZYME

SDH

DPNHD

TPNHD

ESTERASE

AcH

ALK PHOS

ACID PHOS

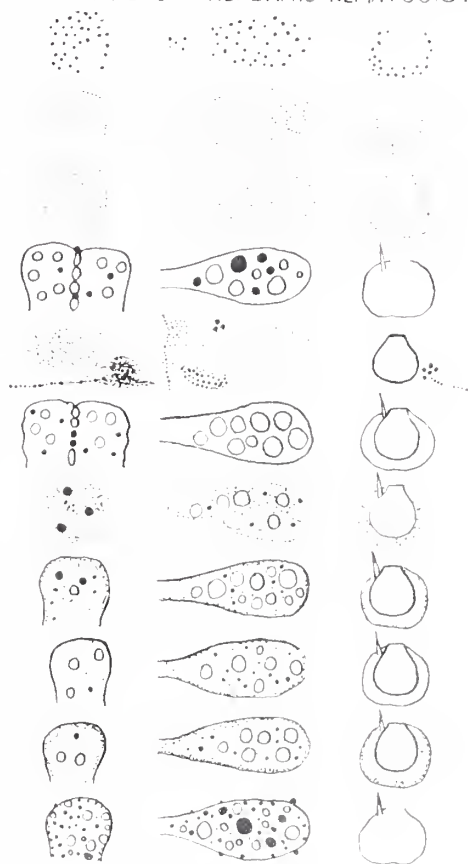
GLUC-6-PHOS

NUCLEOTIDASE

ATP_{ase}

AP

EPIDERMIS GASTRODERMIS NEMATOCYSTS



CYTOLOGICAL LOCALIZATIONS OF ENZYMES

mechanism.

The above suggestion is made even more binding in view of the results of experiments described in Chapter 5. When the tentacles of a hydra are stimulated with a glass rod in the presence of one or more substrates for the critical enzymes, a great number of the mature nematocysts are discharged. If the enzyme substrates are omitted or if inhibitors are added to the substrate medium, virtually no nematocysts are discharged in response to mechanical stimulation.

The possibility of nervous influence on the nematocyst cannot be ruled out entirely. An enzyme activity reasonably similar to mammalian cholinesterase (hydrolyzing acetic acid esters and sensitive to eserine, 62047 and NU-1250) gave histochemical localizations that correspond to the descriptions of the hydra nervous system (Hadzi, '09; Spangenberg and Ham, '60). The activity of this enzyme appeared in cells which correspond in position and shape to those described as ganglion and sensory cells. The fact that the activity was more intense in the hypostome and base could be due to the greater abundance of ganglion cells in these areas.

Concerning the relationship of nervous activity to nematocyst function, the following may be pertinent. If cholinesterase is associated with the transmission of stimuli in the hydra as it is in other metazoan forms,

it would be impossible to rule out neural influences on the control of nematocyst discharge since some of the cnidoblasts are connected by an enzymatically active grape-like cluster of globules to the enzymatically active fibrillar neurites of ganglion cells. Although this arrangement occurred in a spotty fashion throughout the hydra, it occurred mainly in the tentacles. It is probable that this presumed neural connection does not initiate the widespread firing of nematocysts, but this enzymatic and morphological arrangement may instead be concerned with lowering the threshold of excitability of nematocysts in widespread areas. This still leaves the possibility that each nematocyst may be an individual effector.

The localization of acid phosphatase and the oxidative enzymes, succinic dehydrogenase, DPNH and TPNH diaphorase, suggest their distribution in some intracellular organelles in the hydra. Acid phosphatase appeared in all cells as round bodies which resemble mammalian lysosomes in size. Moreover, in some cells many of these structures appeared associated with the cell surface indicating they could be formed by invagination and pinching off of the cell membrane and subsequent distribution as vesicles in the cytoplasm. More of these bodies were seen in the cells of the peduncle, base, and tentacles indicating that they accumulate as cells become older, a phenomenon noted in a variety of mammalian

cells and in tissue culture cells (Novikoff, '61).

Succinic dehydrogenase appeared in granules dispersed throughout the cytoplasm of all cells. These active granules were probably the mitochondria. DPNH diaphorase and TPNH diaphorase, on the other hand, were localized mainly in much smaller granules in the cytoplasm, which were also often attached to the cell membrane. This activity was more abundant in the cells of the hypostome, tentacles, and base. These enzymes are probably located in the endoplasmic reticulum which is sparse in the younger cells of the body but develops as cells differentiate. Only a few of the large granules, presumably mitochondria, which also showed succinic dehydrogenase activity also reacted with these substrates.

The gland cells of the gastrodermis are commonly believed to contain enzymes that are released into the gastric cavity. Non-specific esterase, alkaline phosphatase, acid phosphatase, and aminopeptidase were intensely localized in these cells although the other enzymes were also present. Some of these enzymes could be liberated into the gastric cavity where some extracellular digestion is effected. Digestive cells also possessed many enzymes localized predominantly in cell membranes, membranes of digestive vacuoles, and within the vacuoles (Text Fig. 2). These enzymes among others, thus, could complete intracellularly the digestion of food begun in

the gastric cavity.

The last cytological localization to be considered, a striking and significant one, is the association of all the enzyme activity except succinic dehydrogenase with the cell surface (Text Fig. 2). Such exact and wide-spread occurrence of a number of enzymes in the membranes of mammalian cells has not been observed. Some of the enzyme activity appeared in granules associated with the cell surface while others appeared at the cell membrane. In addition, many of these enzymes were associated with the membranes of intra- and extracellular vacuoles. The finding of enzymes in the plasma membrane should make it possible to study the relationship of enzyme activity to cell permeability in this animal (Chapter 10).

Summary

1. The histochemical localization of several oxidative enzyme systems, some of the carboxylic acid esterases, and some of the phosphatases in the epidermis, gastrodermis, and nematocysts of hydra are reported in this chapter.
2. The tentacles, peduncle, and base were enzymatically specialized in comparison to the hypostome, stomach, and budding region. These histochemical localizations indicate that as cells undergo morphological and functional specialization they undergo enzymatic specialization.
3. Similarly, nematocysts of the tentacles and peduncle,

those that are capable of discharging, are those that are enzymatically specialized. The non-functional nematocysts of the stomach and the budding region are enzymatically unspecialized.

4. The localization of several enzymes in the cnidocil and/or capsule of the nematocyst suggests that enzyme activity, after reception of appropriate chemical stimulation and along with mechanical stimulation, could initiate discharge of the nematocysts.

5. The activity of a cholinesterase was localized in structures that correspond to previous descriptions of the nervous system of hydra. The localization of this enzyme activity in structures connecting the nervous system and nematocysts suggest that this morphological and enzymatic arrangement may be involved in altering the threshold of excitability of nematocyst discharge.

6. Acid phosphatase was localized in structures resembling mammalian lysosomes; succinic dehydrogenase, in mitochondria; and DPNH and TPNH diaphorases, in smaller granules, presumably endoplasmic reticulum. All the enzymes investigated except succinic dehydrogenase were associated with the cell surface.

Legends for Figures

B	base
BR	budding region
Cb	cnidoblast
Cn	cnidocil
DV	digestive vacuole
E	epidermis
G	gastrodermis
H	hypostome
H _M	hypostomal mucous cell
MB	epidermal mucous cell of base
N	nematocyst
P	peduncle
S	stomach
T	tentacle

Explanation of Figures

Fig. 3 Localization of succinic dehydrogenase activity in the epidermal cells of the stomach. Note that only large granular structures, presumably mitochondria, are stained. 1500X

Fig. 4 Isolated nematocysts stained for succinic dehydrogenase activity. Note the reactive large granules in the cytoplasm of the cnidoblasts. Nematocysts show no activity. 1000X

Fig. 5 Low power micrograph of tentacles and hypostome showing the distribution of DPNH diaphorase activity. 100X

Fig. 6 Low power micrograph of hypostome and stomach stained for DPNH diaphorase activity. Note that the cells of the hypostome are more reactive than those of the stomach and that in this region the cell membranes show considerable activity. 100X

Fig. 7 Epidermis of the tentacles showing DPNH diaphorase activity. Note that the activity is primarily associated with cell membranes but also with small and large granules in the cytoplasm. 100X

Fig. 8 Epidermal cells of the stomach showing the localization of TPNH diaphorase activity in small cytoplasmic granules. In the center of the photomicrograph is a cell of unknown character which is very intensely stained. 700X

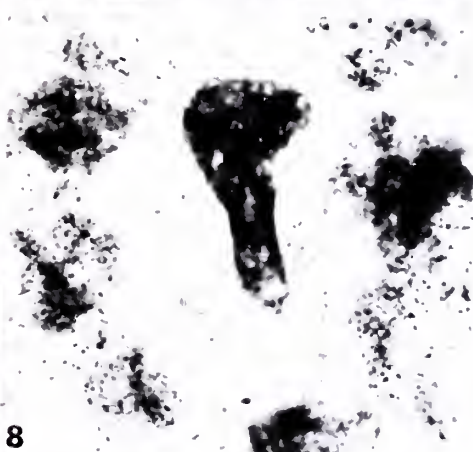
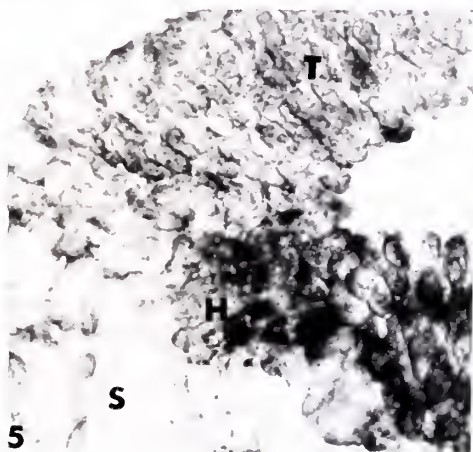
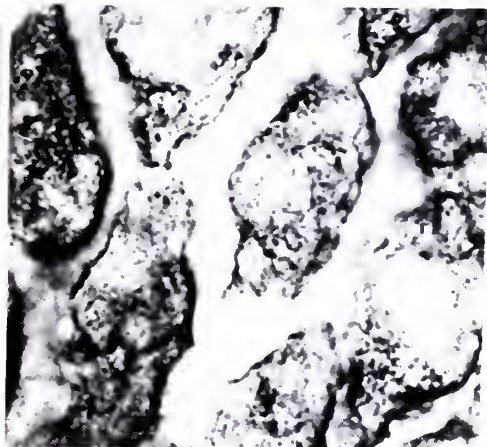
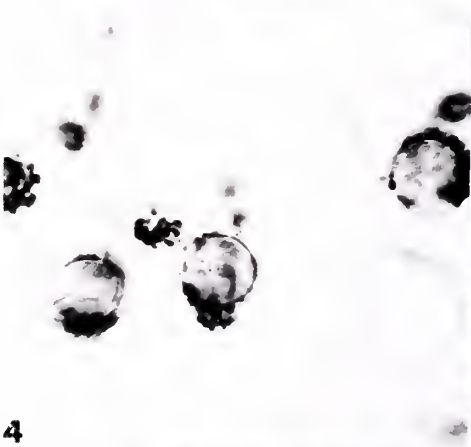
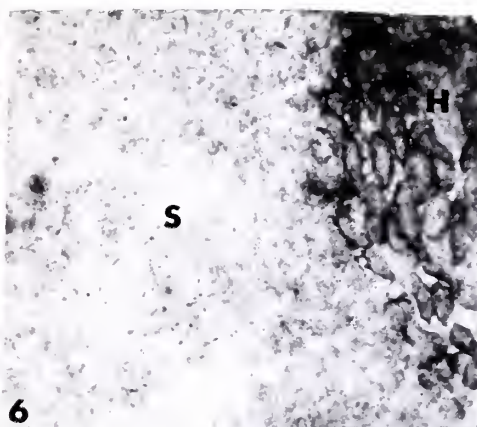
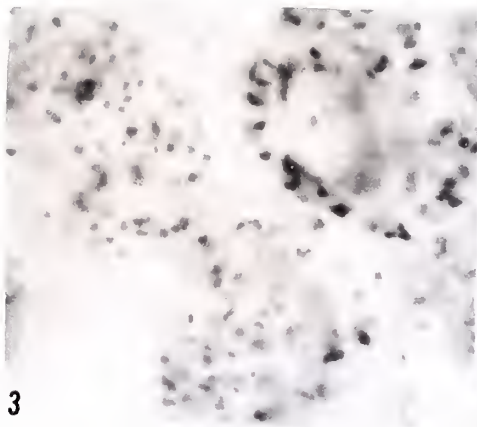


Fig. 9 Nematocyst showing TPNH diaphorase activity. Note reactive granules associated with the cell membrane of the cnidoblast and epidermal cells (arrows). 1500X

Fig. 10 Non-specific esterase activity in the epidermis of the stomach. Enzyme activity is primarily associated with cell surfaces. 400X

Fig. 11 Non-specific esterase activity in the epidermis of the stomach. Most of the activity is confined to the cell surface or intercellular spaces and the arrow indicates active intercellular vacuoles. 1500X

Fig. 12 Epidermis of the stomach region demonstrating cholinesterase activity. Note small granules outlining cell boundaries. Large densely reactive bodies (arrow) are also depicted. 900X

Fig. 13 Cholinesterase activity in cells of the base. Note large reactive bodies (ganglion cells) situated near the bases of the epidermal cells. 400X

Fig. 14 Cholinesterase activity in a large body (ganglion cell) situated in the epidermis of the stomach region. Note the active processes (arrows) that appear to be connected to this cell. 1200X

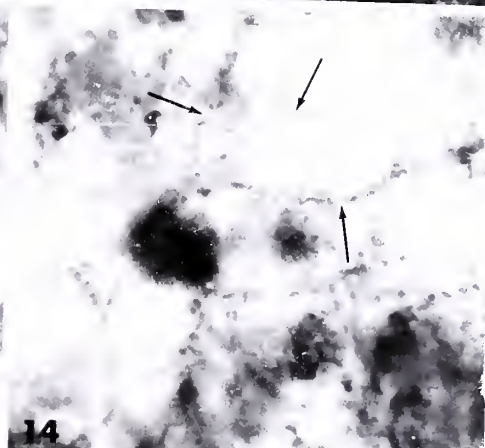
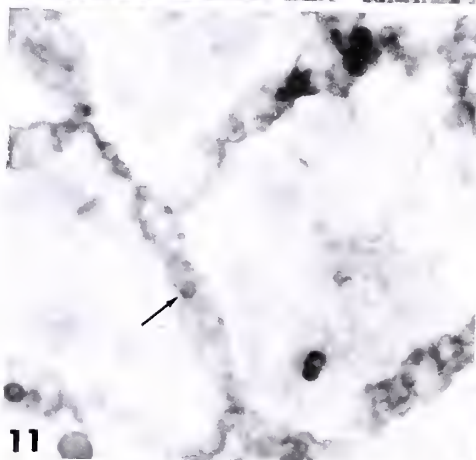
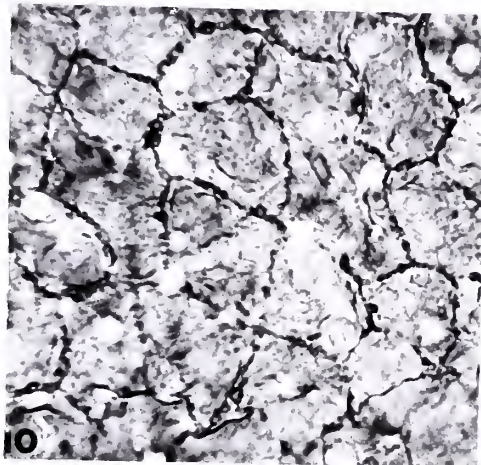
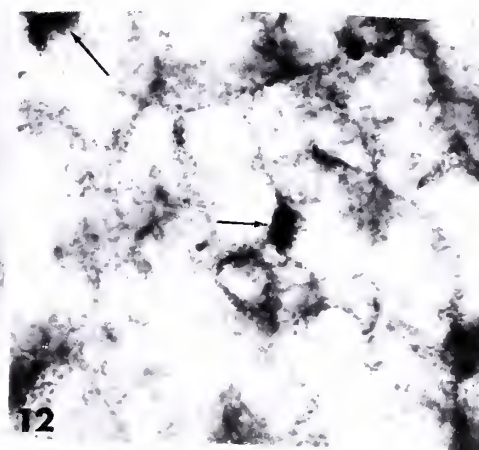
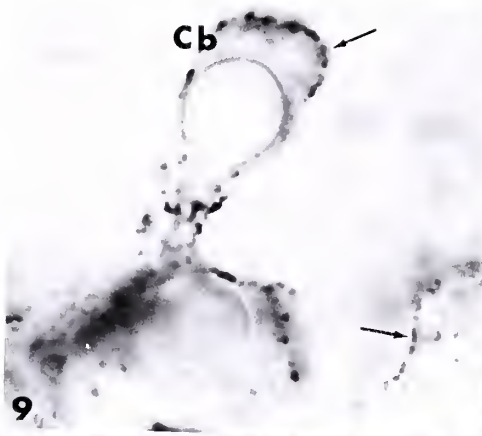


Fig. 15 Cholinesterase active body (presumably sensory cell situated near the surface in the epidermis of the tentacles. 2500X

Fig. 16 Cnidoblasts of the stomach region showing cholinesterase activity localized within the capsules of the nematocysts. 2500X

Fig. 17 Localization of cholinesterase in the tentacles. Note the grape-like clusters (c) of activity associated with the surfaces of the cnidoblasts. In addition, the nematocysts of this region, contrary to those of the stomach region (Fig. 16), show no activity. 1500X

Fig. 18 Cholinesterase activity in a tentacle.

This photomicrograph illustrates similar findings to those in Fig. 17 and in addition demonstrates the enzymatically active processes (arrow) connecting the clusters of activity. 1500X

Fig. 19 Arrows indicate lysosome-like structures that split thiolacetic acid within the cytoplasm of a cnidoclast of the stomach region. 5000X

Fig. 20 Alkaline phosphatase (azodye method) activity in the epidermis of the stomach region. Note activity in cell membranes, in vacuoles between the cells, and in intensely reactive cytoplasmic granules. 600x

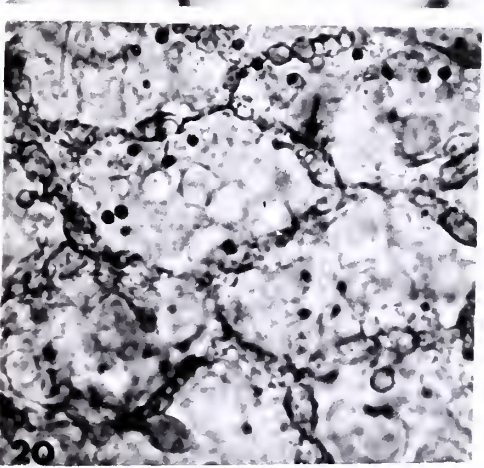
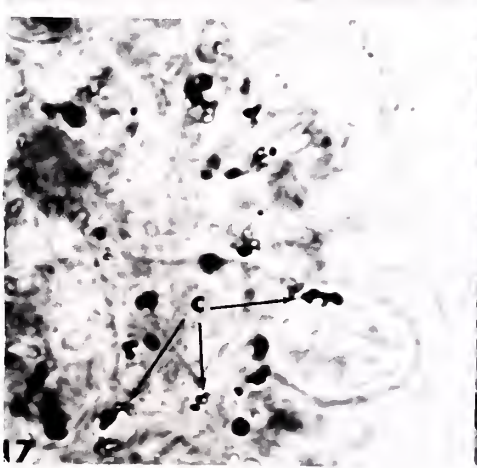
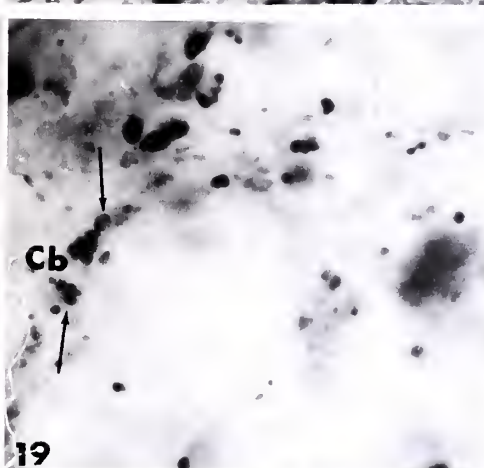
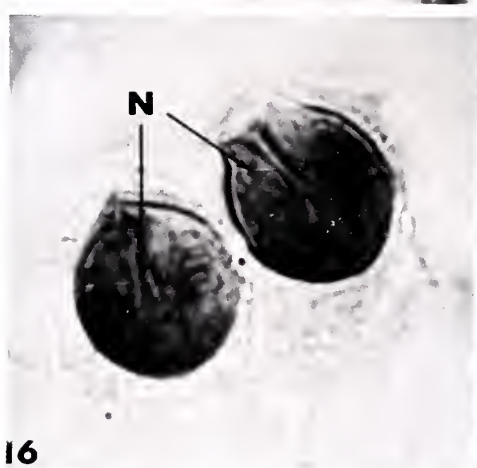
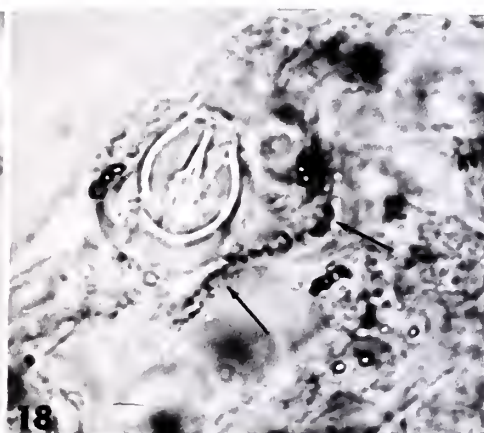
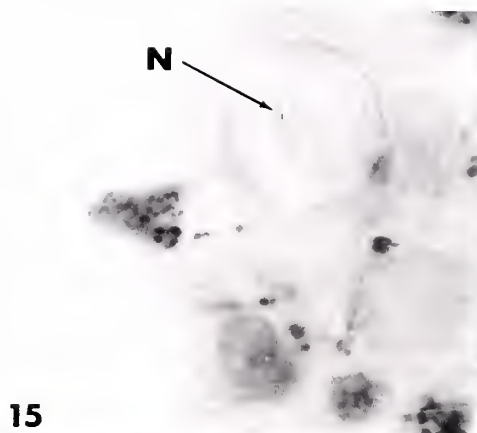


Fig. 21 Localization of alkaline phosphatase activity (metal salt technique) in a gastrodermal digestive cell of the stomach region. Both the cell membranes as well as those of intracellular vacuoles show activity. 1200X

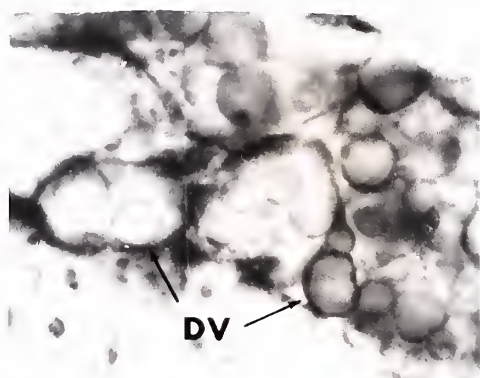
Fig. 22 Nematocysts of a tentacle stained for alkaline phosphatase activity (metal salt technique). Note intense reaction in the capsules and a less intense one in the contents of the nematocysts. 1500X

Fig. 23 Epidermis of the stomach region showing localization of acid phosphatase activity (azo dye technique). Note small granules associated with cell membranes as well as small and large active cytoplasmic granules. 500X

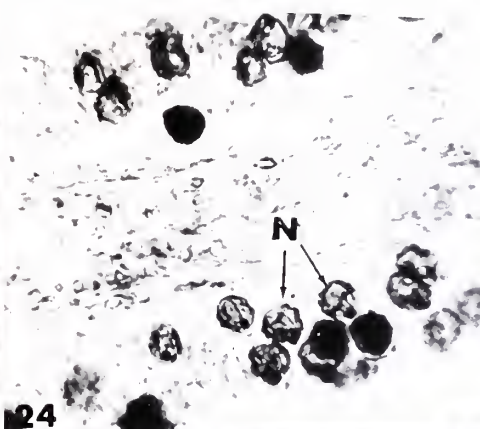
Fig. 24 Section of a tentacle stained for acid phosphatase activity (metal salt technique). Intense activity occurs in capsules and contents of nematocysts. 600X

Fig. 25 Nematocysts of tentacles showing localization of acid phosphatase activity (metal salt technique). Plasma membrane of the cnidoblast, capsule, and contents of the nematocyst as well as cnidocils are stained. 1250X

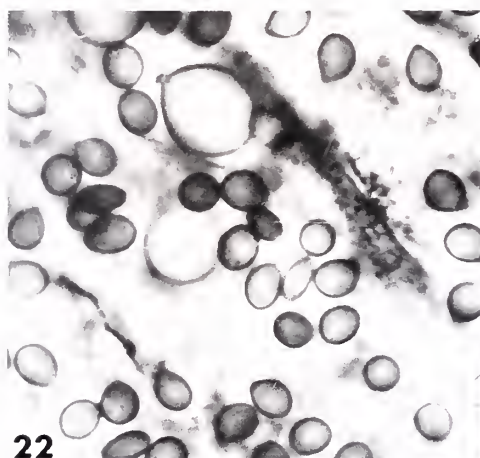
Fig. 26 Gastrodermis of hypostome showing localization of acid phosphatase activity (azo dye technique) primarily within cytoplasmic granules and vacuoles. 350X



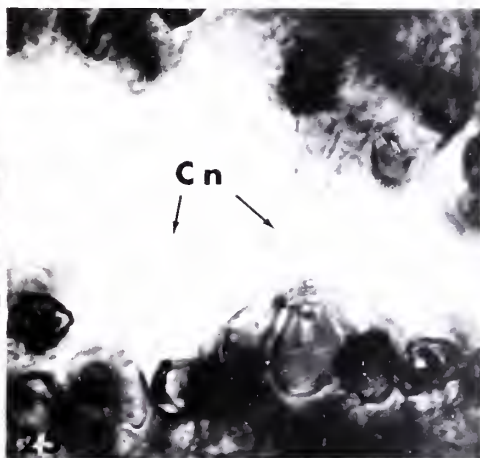
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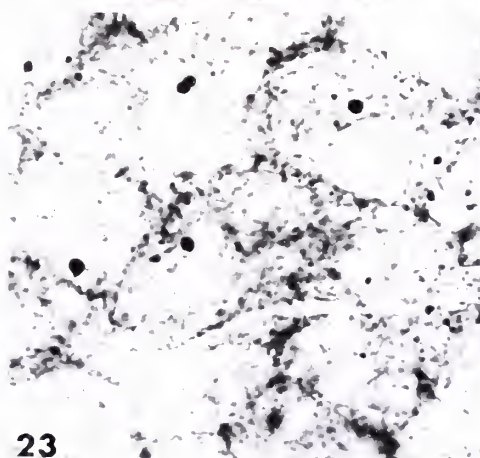
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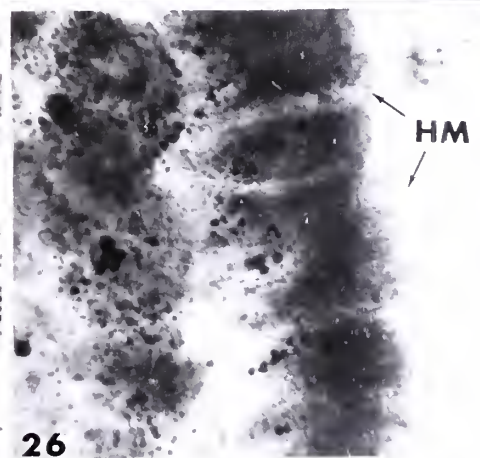
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Fig. 27 Acid phosphatase activity (metal salt technique) in the epidermal cells of the base localized primarily in small cytoplasmic granules. 300X

Fig. 28 Enzyme activity responsible for the hydrolysis of adenosinetriphosphate in the tentacles. Note localization in nematocysts and intense rings of activity at bases of tentacles. 60X

Fig. 29 Epidermis at junction of budding region and peduncle showing localization of enzyme activity responsible for the hydrolysis of glucose-6-phosphate. Note intense area of activity separating the two regions and the activity associated with the cell membranes in the peduncle (arrow). 500X

Fig. 30 Activity of enzyme responsible for the hydrolysis of adenosinetriphosphate in epidermal cells of the peduncle. Note activity associated with the cell surfaces. 900X

Fig. 31 Activity of enzyme responsible for the hydrolysis of adenosine-5'-phosphate in cells of base and peduncle. Note reactive granules outlining cell borders. 500X

Fig. 32 Activity of enzyme hydrolyzing glucose-6-phosphate in section of tentacle primarily localized within nematocysts. 700X

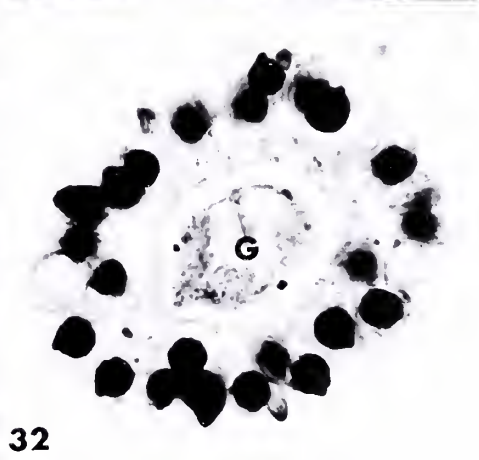
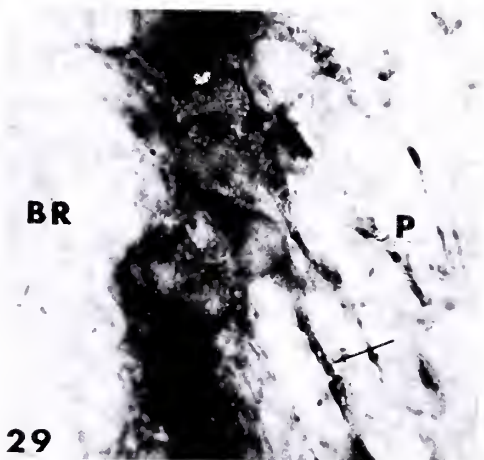
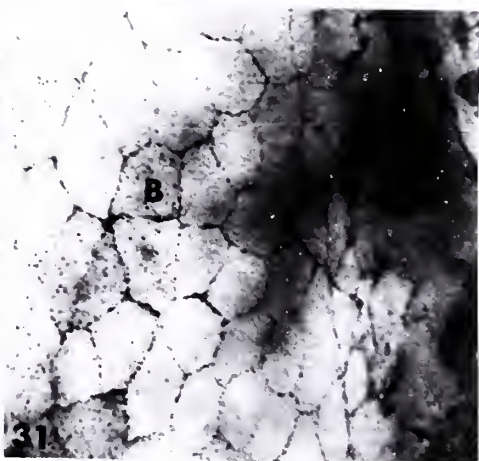
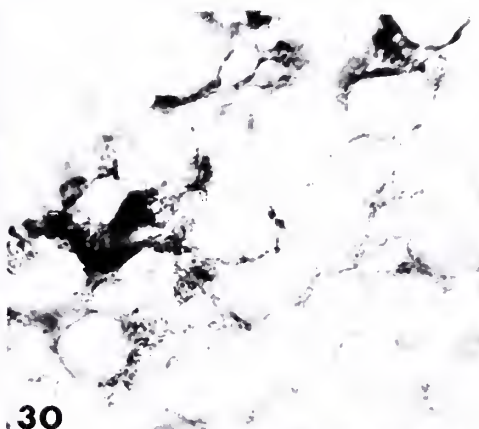
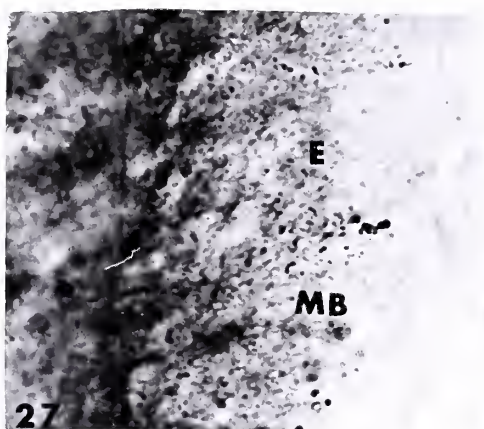


Fig. 33 Digestive cell of gastrodermis showing activity of enzyme hydrolyzing adenosinetriphosphate. Note activity in vacuoles. 800X

Fig. 34 Section through stomach region showing activity of enzyme hydrolyzing adenosinetriphosphate. Note activity in cell membranes and vacuoles of digestive cells. 400X

Fig. 35 Epidermal cells of peduncle stained for aminopeptidase activity. Intensely reactive granules are associated with the cell membrane. 250X

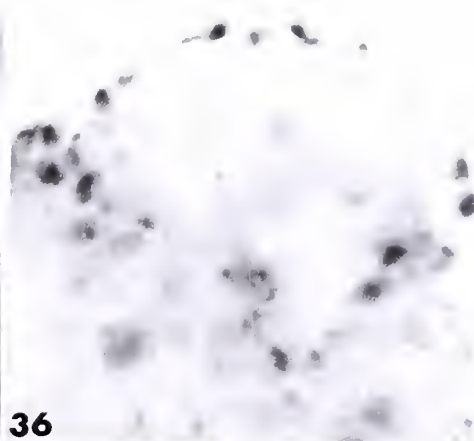
Fig. 36 Epidermal cell of stomach demonstrating aminopeptidase activity. Intensely reactive granules are associated with the cell membrane. 3000X

Fig. 37 Cnidocils showing aminopeptidase activity. 3000X

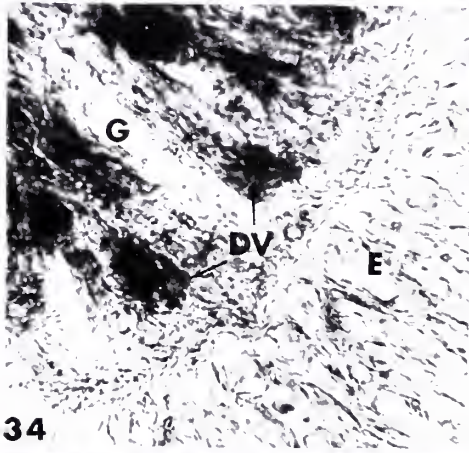
Fig. 38 Localization of aminopeptidase activity in digestive cells of gastrodermis. Note numerous reactive granules and vacuoles. 700X



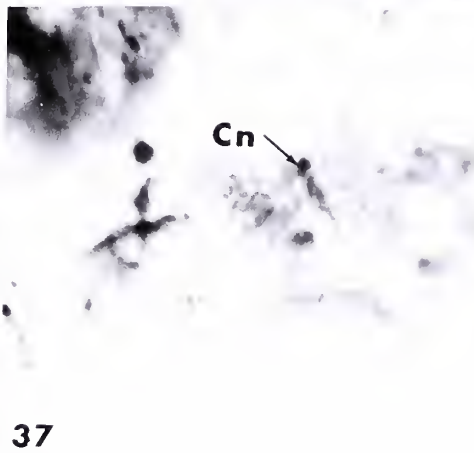
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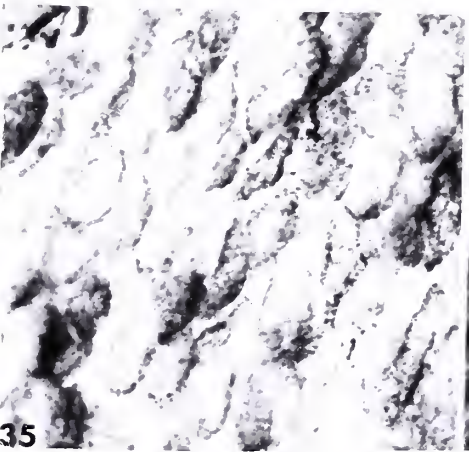
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Chapter 4

Amine Histochemistry of Hydra

Several investigators have used chemical techniques to identify neuropharmacological substances within coelenterates. Welsh ('60, '61) has presented evidence for the presence of 5-hydroxytryptamine (5-HT) and a number of quaternary ammonium compounds in the sea anemone and hydra, the greatest concentration of 5-HT occurring within the tentacles of hydra and the acontia of sea anemones. Methias and his co-workers ('57, '60) identified 5-HT, histamine and other amines in the sea anemone as well as in Physalia. They found the highest concentration of 5-HT occurred in the coelenteric tissues, including the acontia, with relatively low concentration in the tentacles. The possibility was discussed that these compounds were the toxic substances within the nematocysts of sea anemone tentacles; however, the exact histological localization of the substances was not presented.

Recently, histochemical techniques were developed that differentiate epinephrine, norepinephrine, and 5-HT in the same histological section (Wood, '62, '63) and a more sensitive technique was developed for the histochemical detection of norepinephrine (Wood and Barrnett, '63). These techniques were used to localize 5-HT, epinephrine, and norepinephrine in hydra.

Materials and Methods

Intact Hydra littoralis, fixed in 10% formalin or 6% glutaraldehyde (Sabatini et al, '63) were subjected to the histochemical tests and mounted in glycerol jelly. These whole mounts were studied with light microscopy for the observation of the epidermis. The results of the histochemical tests on the gastrodermis were observed on hydra which were turned inside out (Chapter 3).

Observations

All three substances were localized to the nervous system in hydra. Activity was present within ganglion cells, sensory cells, and their neurites. Granular accumulations adjacent to cnidoblasts were active as were a few neurites connecting these accumulations and ganglion cells. No reaction was seen in the nematocysts themselves. These three substances were present in all body regions of hydra, but were most concentrated in the hypostome which contains most of the neural elements (Fig. 1). Epitheliomuscular cells contained a few intensely reactive granules. The nature of these granules is unknown. Digestive cells of all regions possessed a few small vacuoles showing 5-HT activity.

Discussion

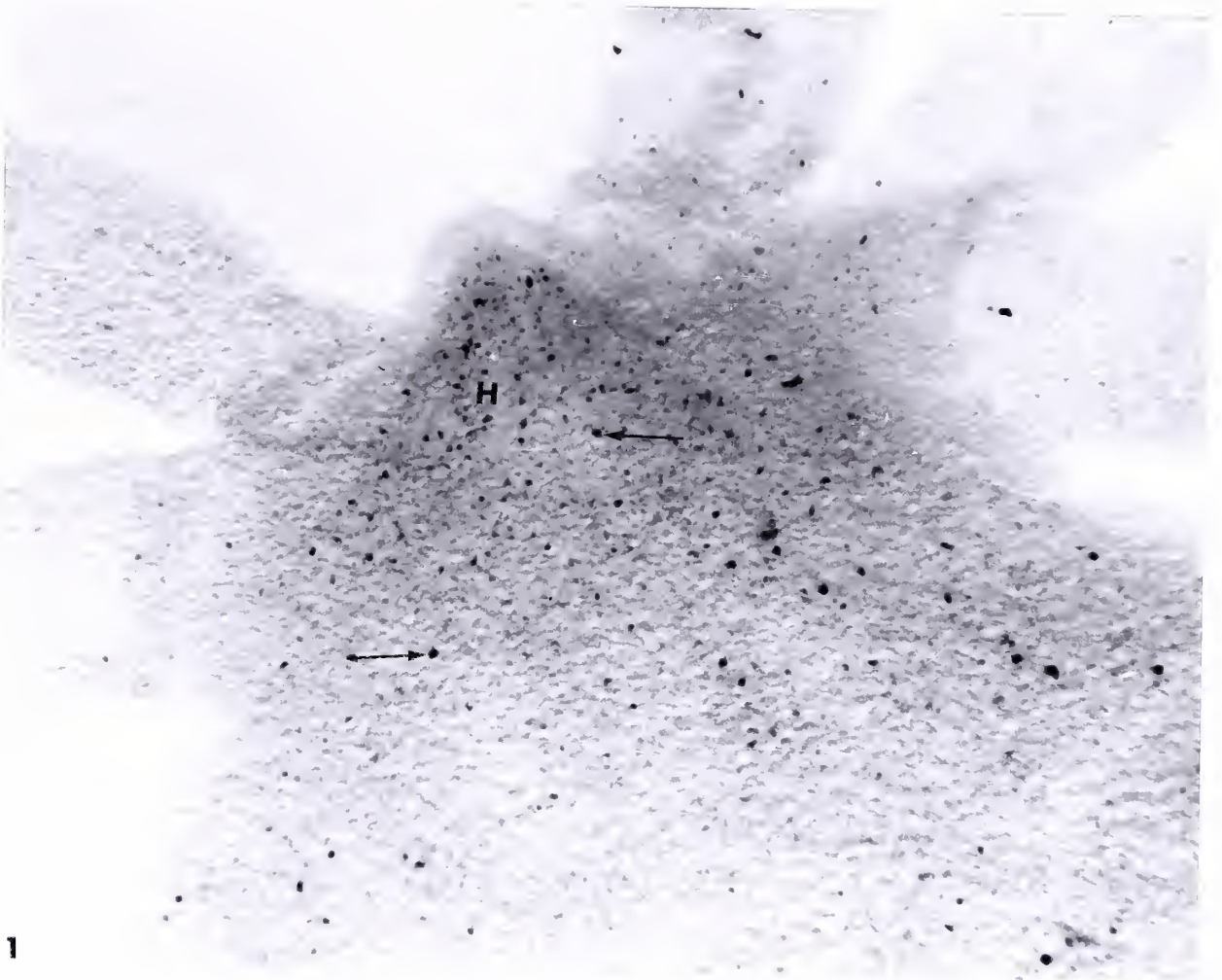
The most surprising aspect of these studies was the presence of epinephrine, norepinephrine, and 5-HT

within the nervous system. The histochemical localization of these substances and the previous identification of acetylcholinesterase (Chapter 3) within the nervous system suggests that an elementary nervous system possesses both adrenergic and cholinergic mechanisms. Furthermore, 5-HT may be of relatively greater importance as a neurohumor in coelenterates than in higher forms. Previous studies have provided evidence that the coelenterate nervous system acting through chemical transmitters may play a role in nematocyst discharge and regeneration. Epinephrine, norepinephrine, 5-HT, and acetylcholine were found to augment nematocyst discharge (Chapter 5). Reserpine which depletes cells of both norepinephrine and 5-HT was a potent inhibitor of regeneration in hydra and various parasympathetic blocking agents had a similar effect (Chapter 7). Thus, the presence of these neurohumors within the nervous system and their effect on physiological processes indicates that the coelenterate nervous system is indeed highly specialized and functions via a variety of neurohumors.

Summary

Epinephrine, norepinephrine, and 5-HT activity was demonstrated in hydra and found chiefly within ganglion cells, sensory cells, and their neurites. These findings suggest that the hydra nervous system possesses adrenergic mechanisms in addition to the previously identified cholinergic.

Fig. 1 Ganglion cells (arrows) in hydra showing norepinephrine activity. Note concentration of these cells in the hypostome (H). 250X



Chapter 5

The Effect of Enzyme Substrates and Pharmacological Agents on Nematocyst Discharge

This chapter is concerned with the functional relationship of the activity of enzymes and of the nervous system to nematocyst discharge in hydra. These highly specialized structures within cnidoblast cells are capable on reception of appropriate stimuli of rapidly discharging a filament that injects a poison and immobilizes or kills the hydra's prey. Four types of nematocysts are recognized in hydra - stenoteles, desmonemes, atrichous isorhizas, and holotrichous isorhizas - and have been studied morphologically by Weill ('34), Hyman ('40), Semal-Van Gansen ('54), Bouillon et al ('58), and Chapman and Tilney ('59a, b). The mechanism and control of nematocyst discharge has been a subject of considerable interest and the majority of recent opinion has suggested that the discharge was primarily a response to a combination of mechanical and chemical stimuli. Early investigators in this area believed control resided in the nervous system.

The content and localization of enzymes in nematocysts (Chapter 3) gave surprising results and these suggested further investigations of the mechanism of nematocyst discharge. Several enzyme activities capable of hydrolyzing common and specific organic phosphoric

acid esters were present in the cnidocil and in the capsule of mature nematocysts contained within the cnidoblasts of the tentacles and peduncle. Furthermore, ganglion and sensory cells and neurites of the hydra nervous system contained an enzyme similar to mammalian cholinesterase. Because of the strategic localizations of enzyme activity in cnidoblasts and because of an intimate connection between some of these cells and the enzymatically active neurites of the nervous system, it was believed that physiological experiments based on the histochemical localization of enzymes would shed considerable light on the mechanism of the control of nematocyst discharge. The effects of enzyme substrates and inhibitors and of neuropharmacological agents on nematocyst discharge are described in this chapter.

Materials and Methods

Hydra littoralis maintained in culture by the method of Loomis and Lenhoff ('56) and modified by Burnett ('59) were used. All experiments were conducted 24 hours after feeding on mature, asexual and non-budding hydra that possessed six tentacles. The basic design of the experiment was to simply place the hydra in the culture medium with or without chemical substrates and reagents and to mechanically stimulate the tentacles by gently stroking with the drawn out end of a glass capillary tube. The hydra were then placed in a drop of water on

a glass slide and a coverslip gently lowered over them. The tentacles of the intact animals were examined with the light microscope (440X) and the number of discharged nematocysts was counted and classified.

In the first series of experiments (controls) the effect of mechanical stimulation of the tentacles in culture medium alone was assayed. These results were compared with those of experiments of similar design in which the hydra was mechanically stimulated in the culture medium containing traces of substrates for some of the enzymes which have been shown to be located in or on the cnidocil, the cnidoblast, or nematocyst (Chapter 3). The substrates and their concentrations are enumerated in Table I.

Acetylcholine was used as a substrate instead of thiolacetic acid which was used in the previous histochemical experiments. Although the latter compound is an excellent substrate for cholinesterase (Wilson, '51), it was not used because of its acidity which alone will cause nematocyst discharge (Jones, '47). In addition, although it was known that several different phosphatases were present in the cnidoblast or the nematocyst, the true identity of these enzymes was not known. Therefore, a wide variety of organic phosphoric acid esters were used as substrates. Other experiments were performed in which the effects of a combination of

substrates together with mechanical stimulation were noted. The enzyme control experiments consisted of the use of mechanical stimulation and both a substrate and an enzyme inhibitor together in the culture medium. The inhibitors and their concentrations are also found in Table I.

In a final series of experiments the effects of mechanical stimulation in the presence of one of several neuropharmacological agents were assayed (Table I). In these experiments, the neuropharmacological agents were used alone, in combination with acetylcholine, in combination with an organic phosphate ester, and in combination with enzyme inhibitors. In each separate treatment several hydra were used and each of these was repeated so that a minimum of 20 hydra were used for each experiment.

Results

The effects of the different reagents and substrates on nematocyst discharge are listed in Table I. The control situation (I,A) of mechanical stimulation in the absence of chemical mediators resulted in virtually no discharge. However, none of the substrates or reagents used in the present experiments caused discharge by themselves without mechanical stimulation. Addition of any one of 10 organic phosphates to the medium (I,B) increased the number of nematocysts that were discharged with mechanical stimulation. While several of the

TABLE I

Effects of Various Reagents on Discharge of Nematocysts

<u>Substances tested</u>	<u>Number discharged</u>			
	<u>Type</u>	<u>of</u>	<u>Nematocyst</u>	
	S	D	AI	HI
A Control - stimulation with glass rod	3	1	3	0
B Cytidine-5'-triphosphate 1/50,000	6	0	9	0
Creatine phosphate 1/50,000	7	0	7	0
Thiamine pyrophosphate 1/50,000	7	1	10	1
Pyridoxal phosphate 1/50,000	22	7	9	1
Adenosine triphosphate (ATP) 1/50,000	12	10	19	1
Glycerophosphate 1/50,000	16	14	13	1
Adenosine-5-phosphate (AMP) 1/50,000	12	32	22	0
Glucose-6-phosphate 1/50,000	83	23	13	0
Fructose-6-phosphate 1/50,000	175	10	21	2
Fructose-1,6-diphosphate 1/50,000	161	2	1	0
C Glycerophosphate 1/50,000 + NaF ($10^{-2}M$)	1	0	0	0
Glycerophosphate 1/50,000 + N-ethyl maleimide $2 \times 10^{-4}M$	2	4	2	0
AMP 1/50,000 + Ni^{++} ($10^{-2}M$)	8	9	2	0
AMP 1/50,000 + ethyl maleimide ($2 \times 10^{-4}M$)	3	6	2	1
ATP 1/50,000 + phloridzin ($10^{-2}M$)	8	4	3	0
ATP 1/50,000 + N-ethyl maleimide ($2 \times 10^{-4}M$)	2	4	3	2
Glucose-6-phosphate + NaF ($10^{-2}M$)	1	0	0	0
Glucose-6-phosphate + ethyl maleimide ($2 \times 10^{-4}M$)	4	6	2	0
D Acetylcholine (ACh) 1/100,000	105	30	17	1
Eserine ($10^{-5}M$)	146	58	11	0
ACh 1/100,000 + hexamethonium 1/100,000	3	2	2	0
Hexamethonium 1/20,000	3	3	1	0
Tubocurarine 1/20,000 + ACh 1/100,000	9	2	2	0
Decamethonium 1/100,000	45	2	9	1
Decamethonium 1/10,000	281	10	10	0
Atropine 1/50,000	74	5	7	0
Atropine 1/50,000 + ACh 1/100,000	152	29	25	0
E Glycerophosphate 1/50,000 + ACh 1/100,000	22	11	10	0
AMP 1/50,000 + ACh 1/100,000	26	4	12	0
ATP 1/50,000 + ACh 1/100,000	31	14	14	0
Glucose-6-phosphate 1/50,000 + ACh 1/100,000	100	5	11	0
F ACh 1/100,000 + N-ethyl maleimide ($2 \times 10^{-4}M$)	20	15	5	0
ACh 1/100,000 + NaF ($10^{-3}M$)	25	9	15	1

Fructose-6-phosphate 1/50,000 + hexamethonium 1/20,000					195	49	8	0
Glucose-6-phosphate 1/50,000 + hexamethonium 1/20,000					72	6	2	0
G	Epinephrine 1/10,000				25	25	13	10
	Glucose-6-phosphate + epinephrine				83	9	12	0
	5-hydroxytryptamine 1/10,000				37	10	24	7
	Glucose-6-phosphate + 5-hydroxytryp- tamine				77	12	23	4
	Norepinephrine 1/10,000				37	54	170	5
	Histamine 1/10,000				90	55	107	9

substrates caused a slight ($B_{1,2,3}$) or a moderate ($B_{4,5,6,7}$) increase in the number of nematocysts discharged, three substrates ($B_{8,9,10}$) were associated with massive discharge. It should be noted that in the cases of the hexose phosphates, discharge was largely confined to stenoteles, and none of the substrates had an appreciable effect on holotrichous isorhizas.

Enzymatic inhibitors (I,C) decreased the number of nematocysts ordinarily discharged in the presence of the substrates alone. Sodium fluoride proved to be the most efficient inhibitor ($C_{1,7}$) and although N-ethyl maleimide, a sulfhydryl-blocking reagent, should have affected only some of the phosphatases (e.g. adenosinetriphosphatase but not alkaline or acid phosphatase) it had a general inhibiting effect ($C_{2,4,6,8}$). This might indicate that a sulfhydryl-dependent enzyme system was associated with the discharge mechanism.

In assessing the effect of the nervous system on nematocyst release, it was found that acetylcholine (D_1) caused massive discharge. This was augmented by eserine (D_2), and inhibited by hexamethonium ($D_{3,4}$), and by tubocurarine (D_5). Decamethonium ($D_{6,7}$) produced excessive discharge, especially of stenoteles. Atropine (D_8) caused an increased release of nematocysts and augmented the release produced by acetylcholine (D_9). Acetylcholine, together with one of the phosphate substrates (I,E)

produced a discharge greater than that of the phosphate alone, but less than that of acetylcholine alone. As before, the release involved especially stenoteles, as well as desmonemes and atrichous isorhizas but not holotrichous isorhizas. Enzyme inhibitors, exclusive of cholinesterase inhibitors ($F_{1,2}$) seriously decreased the response that was obtained with acetylcholine but hexamethonium did not alter the discharge produced by enzyme substrates ($F_{3,4}$).

Both epinephrine (F_1) and 5-hydroxytryptamine (F_3) caused a moderate release of nematocysts. Norepinephrine (F_5) and histamine (F_6) caused massive release. These amines were the only agents to affect holotrichous isorhizas and the latter two caused by far the greatest discharge of atrichous isorhizas. Addition of a phosphate substrate did not appear to augment the release produced by amines ($G_{2,4}$).

Discussion

Numerous studies have been performed on the control and mechanism of nematocyst discharge (see Hyman, '40, '59, for review). The earliest view (Chun, 1881; Lendenfeld, 1887; Murbach, 1893; Grenacher, 1895) assumed that the firing of nematocysts was dependent on the nervous system. A later view, which has now gained wide acceptance, suggested that the nematocysts are independent effectors which require a combination of chemical and

mechanical stimulation for eversion (Iwanzoff, 1896; Parker and Van Alstyne, '32; Pantin and Pantin, '42; Pantin, '42a, b; Ewer, '47; Jones, '47; Picken, '53; Robson, '53; Burnett, Lentz, and Warren, '60). These investigators found that either mechanical or chemical stimulation (food extracts) alone failed to produce discharge. Since the combination of the two stimuli resulted in firing, it was believed that nematocysts responded to mechanical stimulation when the threshold for eversion had been lowered by the chemical stimulation. However, nematocysts do not discharge in anesthetized animals (Glaser and Sparrow, '09; Jones, '47; Glumac, '53; Pantin, '56), and this suggested that the mechanism of discharge lay in a neural circuit contained within each individual cnidoblast, assuming the individual effector hypothesis.

The histochemical experiments (Chapter 3) suggested that enzymatic activity may play a role in nematocyst discharge. The present experiments provide further evidence for this view. Mechanical stimulation in the presence of an enzyme substrate resulted in augmented discharge of nematocysts, whereas mechanical stimulation alone, or in the presence of an enzyme inhibitor as well as the substrate, produced virtually no discharge. Thus, it appears that mature nematocysts containing strategically placed enzymes are able to respond (providing mechanical stimulation also occurs) to chemical reagents which serve

as enzyme substrates and therefore as effector substances. These findings indirectly support the independent effector hypothesis.

Although in the present case nematocyst discharge appeared to be related to the activity of one or more phosphatases, it would not be surprising if a variety of substrates for other enzymes located in cnidoblasts might also augment the discharge. Toward this end, it has been found that leucyl-naphthylamide which is hydrolyzed by one or more aminopeptidases produced a discharge greater than most of the phosphate substrates. In addition, the identity of the phosphatases concerned with the enzyme splitting are not known. For example, glucose-6-phosphate served as quite a satisfactory substrate for nematocyst discharge and for the histochemical demonstration of activity but the enzyme that split it was quite different histochemically from rat liver glucose-6-phosphatase. If there is a correlation between the effectiveness of a substrate and nematocyst discharge, at least the presence of an hexose phosphatase which shows a degree of enzyme specificity for substrates esterified in the 6 position would have to be postulated.

However, a neural influence on nematocyst discharge cannot be ruled out for several reasons. From a morphological point of view, several investigators have described the occurrence of nerves terminating on cnido-

blasts (Chun, 1881; Lendenfeld, 1887; Murbach, 1893; Kepner et al, '43; Spangenberg and Ham, '60). From a histochemical point of view, it was found (Chapter 3) that the linear extracellular fibers connecting ganglion cells and terminating on the surface of cnidoblasts showed cholinesterase activity. It should be noted, moreover, that although all cnidoblasts contained some intracellular cholinesterase activity, the termination of enzymatically active neurites on cnidoblasts occurred in a spotty fashion.

This morphological and enzymatic arrangement led to a series of assays in which the effects of a variety of neuropharmacological agents on nematocyst discharge were tested. Acetylcholine produced a considerable discharge especially of stenoteles and this was augmented by eserine, a cholinesterase inhibitor. Hexamethonium and tubocurarine eliminated the effectiveness of acetylcholine. Decamethonium increased nematocyst discharge because of its well-known action as a depolarizer. The fact that atropine also caused a discharge was a surprising finding. It is suggested that this reaction may be non-specific and the fact that atropine did not block the discharge suggests that the innervation of nematocysts is preganglionic in type. It should be recalled that preganglionic impulses are blocked by hexamethonium and neuromuscular impulses are blocked by tubocurarine but neither is blocked by atropine which blocks postganglionic impulses. In this re-

gard the innervation of some nematocysts (e.g., stenoteles) can be considered similar to the somatic efferent innervation of ganglion cells. On the basis of this evidence, it appears highly probable that the nervous system of hydra plays a significant role in nematocyst discharge. This suggestion is made even more binding since several neurohumoral amines, epinephrine, norepinephrine, 5-hydroxytryptamine and histamine also augment discharge. The fact that the amines particularly affected both types of isorhizas might suggest that the neural mechanism in these nematocysts was different from that in the stenoteles.

Either a combination of mechanical and chemical stimulation or mechanical and nervous stimulation effects a discharge of nematocysts. The chemical stimulation appeared related to the activity of enzymes. The exact site of the enzymes is not known but the clear implication is that they are on the surface of the cnidocil or are present in the cnidocil or the capsule of the nematocyst, localizations borne out by the histochemical studies (Chapter 3). The enzyme activity, if located in the interior of the cnidoblast, may be concerned with a chain of metabolic events from mechanical stimulation of the cnidocil to the discharge of the nematocyst. Chemical stimulation did not require a functional nervous system since the substrate-augmented response was not blocked by

hexamethonium. On the other hand, nervous system stimulation also appears to have an excitatory effect on nematocyst discharge even in the absence of exogenous enzyme substrates. However, the combination of chemical and nervous stimulation is additive and this should not be surprising since it is a fairly common phenomenon that two stimulating agents frequently do not augment each other, especially if they react at different sites in a chain of complex events. In addition, the nervous system appears to act through metabolic enzyme systems which may be the intrinsic final common pathway since enzyme blockers decrease the response to nervous stimulants.

On this basis, these results could be interpreted that each of chemical and nervous stimulation augments or facilitates the process with which mechanical stimulation effects the discharge of nematocysts. Although each stimulus by itself appears to be subliminal, a combination of two stimuli, one of which is mechanical, pool to effect a response. Furthermore, each type of stimulus appears to be a different subliminal mechanism in which chemical stimulation does not effect the nervous system and the latter does not effect chemical stimulation, but all stimuli including mechanical appear to act through a chain of enzymatic events. The independent effector hypothesis for discharge of nematocysts thus appears to be a correct one, since a minimum of two stimuli, one of

which is local and mechanical, appears to be required. This does not mean that the stage cannot be set by diffuse chemical stimuli acting through enzymes or by widespread nervous discharge effecting those cnidoblasts that are innervated or that are effected by neurohumors.

Summary

The effects of enzyme substrates and inhibitors and of neuropharmacological agents on nematocyst discharge are reported in this chapter.

1. The control situation of mechanical stimulation of the tentacles in the absence of chemical mediators resulted in virtually no nematocyst discharge.
2. Addition of organic phosphates to the medium produced nematocyst discharge with mechanical stimulation. Enzymatic inhibitors practically eliminated the discharge produced in this manner.
3. In regard to the nervous system, acetylcholine produced a massive discharge which was augmented by eserine and inhibited by hexamethonium and tubocurarine.
4. Enzyme inhibitors, exclusive of cholinesterase inhibitors, decreased the response that was obtained with acetylcholine but hexamethonium did not alter the discharge produced by enzyme substrates.
5. Epinephrine, 5-hydroxytryptamine, norepinephrine, and histamine caused release of nematocysts and were the only agents to affect holotrichous isorhizas.

6. These experiments indicate that mature nematocysts containing strategically placed enzymes are able to respond (providing mechanical stimulation also occurs) to chemical reagents which serve as enzyme substrates and therefore as effector substances. In addition, the nervous system of hydra appears to play a significant role in nematocyst discharge.

7. Each type of stimulus appears to be a different subliminal mechanism in which chemical stimulation does not affect the nervous system and the latter does not affect chemical stimulation, but all stimuli including mechanical appear to act through a chain of enzymatic events.

Chapter 6

Changes in the Distribution of Enzymatic Activity in the Regenerating Hydra

Hydra possess remarkable regional histochemical localization of some enzyme activities (Chapter 3). The epidermal cells of the tentacles, peduncle, and base contain an intense concentration of several different hydrolases, especially phosphatases, in contrast to the cells of stomach and budding regions which possess little activity. In addition, cnidoblasts and/or nematocysts of the tentacles or peduncle possess a variety of enzyme activities, whereas the same cells in the hypostome, stomach, budding and growth regions show none of these activities. These observations lead to the assumption that as cells move from the growth region toward the tentacles or base and become morphologically and functionally developed, they also become enzymatically specialized. It was also noted during the histochemical studies on the hydra that small buds protruding laterally from the budding region possessed histochemical distribution similar to that of the budding region whereas fully formed and separated buds possessed the same distribution of enzyme activity as mature buds.

Changes in the intensity and distribution of histochemically demonstrable enzymatic activity might be expected to take place during cell differentiation in a

regenerating portion of hydra. The peduncle and base were chosen as the areas of hydra to study enzyme dedifferentiation and differentiation, since these normally are a highly morphologically and enzymatically specialized portion. The changes in histochemical localization of enzymatic activities undergone by the peduncle and base during its regeneration into a complete animal are described.

Materials and Methods

Hydra littoralis cultured in this laboratory by the method of Loomis and Lenhoff ('56) modified by Burnett ('59) were used. The peduncle and base of mature hydra was severed just below the budding region, removed, and allowed to regenerate in fresh media without feeding. After one, two, three, four, and five days of regeneration, either fresh or formol-calcium fixed regenerates as whole mounts were subjected to the enzyme histochemical tests and observed with ordinary light microscopy. The histochemical tests included acid phosphatase (Gomori, '50), alkaline phosphatase (Gomori, '52), 5'nucleotidase (Wachstein, '55), adenosine-triphosphatase (ATPase) (Wachstein, '57), glucose-6-phosphatase (Wachstein and Meisel, '56), succinic dehydrogenase (SDH) (Nachlas et al, '57) and reduced diphosphopyridinenucleotide (DPNH) diaphorase. These methods and their application to hydra are described completely in Chapter 3.

Observations

Before considering the results of the histochemical tests on the regenerating peduncle and base, the regional localizations of enzymatic activity in the intact hydra should be reviewed. Acid and alkaline phosphatase, as well as the special phosphatases (5'-nucleotidase, ATPase, and glucose-6-phosphatase) were intensely reactive in the epidermal cells of the tentacles, peduncle, and base. The epidermal cells of the hypostome, stomach, and budding region were much less reactive. These enzymes were also localized in or on the cnidocil and the capsules of nematocysts, but only those of the tentacles and peduncle. DPNH diaphorase activity was present in all regions of hydra but the cells of the tentacles, hypostome, peduncle, and base showed a reaction that was slightly more intense than the other parts. SDH activity was evenly distributed in the epidermal cells throughout the hydra (Chapter 3).

Regeneration of the amputated peduncle and base into a complete hydra was accompanied by profound alterations in the distribution and intensity of some of the enzymatic activities. Three characteristic patterns of change were observed for the phosphatases and these results were quite different from the findings for the oxidative enzymes. The epidermal cells of the normal peduncle and base by and large showed acid phosphatase activity. Within this region a zone of cells containing very intense activity

occupied the distal portion of the peduncle adjacent to the growth region. The cells of the base were also intensely reactive but the cells of the intervening portion of the peduncle showed somewhat less activity (Fig. 1). The enzyme was localized in epithelial cells in small cytoplasmic vesicles, presumably lysosomes, some of which are attached to cell membranes, in intracellular vacuoles, and in the capsule of nematocysts. After one day of regeneration, the cells of the regenerate were strikingly less reactive, especially at both the base and distal end (Fig. 2). The reactive cytoplasmic vesicles and vacuoles are much less numerous. Nematocyst capsules, however, retained their activity. The small amount of enzymatic activity after two days of regeneration was confined to a few vesicles in epidermal cells and to the nematocysts. Activity first reappeared after three days of regeneration in the distal ends of the developing tentacles. In this region the nematocysts possessed the intense capsular activity of mature nematocysts. In the remainder of the hydra, only scattered nematocysts showed activity and the epidermal cells were unreactive (Fig. 3). The reactive nematocysts in the body of the regenerate probably represent ones which have migrated from the original peduncle, for it was only under the circumstance of regeneration that active nematocysts were found in this region. This observation suggests a difference between nematocysts and

epidermal cells; mature nematocysts of the peduncle remained mature and did not lose their activity while the epidermal cells lost activity during regeneration. The distribution of enzyme activity of the normal hydra was partially attained after four days of regeneration. The tentacles of the regenerate were the most reactive region and the differentiating peduncle showed signs of increasing activity. Nematocysts in these two regions were the most reactive cell type, but a little activity occurred in epidermal cells. The nematocysts in the tentacles and peduncle probably represent newly matured ones since this observation conforms to the previous ones (Chapter 3) that nematocysts only gain activity when they have migrated to these regions. The newly-formed base contained no activity (Fig. 4). At day five increased numbers of reactive nematocysts in the tentacles and peduncle and increased numbers of vesicles in epidermal cells of the tentacles, peduncle, and base gave the hydra an enzymatically mature appearance for this enzyme.

Intense alkaline phosphatase activity occurred rather homogeneously in the cells of the peduncle and base (Fig. 7) and was localized to the capsules of nematocysts, membranes of epidermal cells, and in the membranes of intra- and extra-cellular vacuoles. After one day, the rapidly growing region of the regenerate showed a less intense reaction, but in the remainder of the animal the

intensity and the localization of the enzyme activity remained approximately the same as it was in the intact animal (Fig. 8). The intense activity seen in the normal peduncle returned to the entire regenerate except for the base after two or three days (Fig. 9). Thereafter, the activity of all parts remained at a high level throughout regeneration (Fig. 10).

The special phosphatases, 5'-nucleotidase, ATPase, and glucose-6-phosphatase, so named because of the substrate used in each case, are considered together because of their similar cytological localizations in the mature hydra. The changes of localization for these enzymes differed from those of acid and alkaline phosphatase. Enzymatic activity for 5'-nucleotidase and glucose-6-phosphatase were the same but those of ATPase differed to some extent. In the normal peduncle which was intensely reactive, all three enzymes were localized in cell membranes, tiny granules attached to the cell membranes, and in the capsules of nematocysts. The cells of the base had similar but fewer sites of activity (Figs, 13, 17). One day after the peduncle was removed, practically all 5'-nucleotidase and glucose-6-phosphatase activity was lost (Figs. 5, 14). Only the cell membranes and a few associated granules were reactive and nematocysts were unreactive at this stage. With ATPase some activity persisted in membranes of epidermal cells and nematocysts of the peduncle but the cells

of the base remained strongly reactive (Fig. 18). At day two, intense activity for all three enzymes reappeared at the distal portion where the tentacles were being formed. The remainder of the regenerate showed weak activity for 5'-nucleotidase and glucose-6-phosphatase and moderate activity for ATPase. The latter enzyme was particularly decreased in cells of the base. After three days of regeneration, the tentacles showed intense activity for all three enzymes localized to nematocysts and to epidermal cells. In the remainder of the regenerate scattered loci of cells and a few nematocysts showed 5'-nucleotidase or glucose-6-phosphatase activity (Fig. 15). ATPase activity was also at these sites but in addition many of the cells of the body of the regenerate except for the base showed moderate activity (Fig. 19). By four or five days of regeneration, intense activity for 5'-nucleotidase and glucose-6-phosphatase was noted in the tentacles primarily confined to nematocysts (Figs. 6, 16). At the distal portion of the hydra, epidermal cells and nematocysts of the peduncle first showed reconstitution of activity for these enzymes (Fig. 16) and by the fifth day the cells of the base were also active (Fig. 6). As the epidermal cells and nematocysts of the tentacles gained ATPase activity in the fourth and fifth day of regeneration, the activity in the remainder of the regenerate disappeared (Fig. 20). Once that had occurred, activity in the cells of the

peduncle and base was reconstituted.

DPNH diaphorase activity was normally localized in tiny cytoplasmic granules, presumably endoplasmic reticulum, while SDH activity was present in larger granules, presumably mitochondria, in the epidermal cells and cnidoblasts. No changes in cytoplasmic localization or intensity of reaction of these enzymes could be detected during regeneration (Figs, 11, 12, 21, 22).

Discussion

The elucidation of metabolic or enzymatic capacities of regenerating cells should lead to a better understanding of differentiation. This hypothesis is really an oversimplification because it merely states that a cell as a structural and functional entity is limited by its enzymatic content. As pointed out by Moog ('59), in order to translate enzymology into causal analytical mechanisms, one must demonstrate the structural orienting mechanisms by which cells manipulate the enzymes they contain. From this point of view the present work has simply demonstrated that hydra cells can manipulate some of their enzyme contents to fit a variety of circumstances concerned with growth and differentiation. The most striking finding was the modulation of the activities of different phosphatases during regeneration. These findings are particularly significant in relation to the regional localization of enzymes in the normal hydra (see Introduction).

The most immediate enzymatic change noted after amputation of the base and peduncle was a decrease in the activity of the phosphatases and a change in their distribution. While this was marked and prominent for 5'-nucleotidase, glucose-6-phosphatase, and acid phosphatase, the loss of ATPase was less marked and delayed and alkaline phosphatase decreased only slightly. Accordingly this initial reaction may be called the stage of enzyme dedifferentiation since it implies a loss in enzymatic specialization from highly specialized cells before regeneration proceeds. Stated another way, the cells of the peduncle and base lose their functional (enzymatic) specialization as they change from a normal situation to one in which rapid growth will become the major expression.

This observation is not consistent with Burnett's ('61) conclusion that regeneration of the peduncle of hydra is due to cellular division of interstitial cells in the distal region of the peduncle and their later differentiation into other cell types. Although the proliferation of new cells appears not to be involved in the initial regulation and regeneration of hydroid polyps (Cowden and Glocker, '60), Burnett furthermore indicated that there was no evidence that a specialized cell type was capable of dedifferentiating into an interstitial cell. Furthermore, if the interstitial cells are selectively destroyed by X-radiation, hydra is incapable of

regeneration (Zawarzin, '29; Evlokhova, '46; Brien, '53, '55). Thus, an increase in the number of interstitial cells, unspecialized morphologically and enzymatically, accounts for at least some of the decreased enzyme activity in the regenerating peduncle. However, it should be noted that the entire peduncle lost its activity for some enzymes after one and two days of regeneration, not merely at the distal end where interstitial cells were accumulating.

Potential reasons for these findings can be hypothesized in broad conceptual terms and can later be experimented upon. At the present stage of this investigation, it appears that the hydra like other regenerating forms, be it the tail of the earthworm or mammalian liver, receives information when regeneration has proceeded to the stage of biologic completeness. Prior to this stage and lacking the information, the hydra concentrates on growth and form and as a result most cells attain a premature functional state even though only some of them (interstitial) are concerned with growth.

During the period of active growth, the distribution of enzymatic activity in the regenerate was quite similar to that occurring in the growth region of the normal intact hydra or that occurring in a small lateral bud. This similarity supports the hypothesis mentioned above and may signify that growth regions whether in

intact hydra or in regenerates have a different enzymatic pattern than the other areas of the intact hydra which have become specialized morphologically and functionally for other duties.

The disappearance of acid phosphatase containing bodies (lysosomes) in epidermal cells during regeneration and the beginning reappearance of these bodies after regeneration is important. In the first place, it is in agreement with the suggestion of Novikoff ('61), who indicated that the presence of these enzyme-containing structures may be a function of the age of some cells, appearing only in older, differentiated cells whereas younger differentiating cells contain few, if any. This suggestion is intimately related to a second and more important hypothesis which is based on the fact that the acid phosphatase method was used as a marker to demonstrate these bodies (lysosomes) which actually contain a variety of destructive hydrolases and cathepsins (DeDuve, '59). The decrease in acid phosphatase activity therefore may be taken as a sign that there is an overall decrease in catabolic activity during regeneration. Although this statement may have some teleological implications, in order for growth and differentiation to occur anabolic events must exceed those of the catabolic ones and it is indeed fortuitous that the experiments indicate a virtual disappearance of the catabolic bags of enzymes (lysosomes)

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while the hydra regrows.

Alkaline phosphatase activity, on the other hand, remained at a fairly high level during the regenerative process. Similar but less striking findings were found for ATPase. Alkaline phosphatase activity was stronger in the hypostome, growth region, stomach, and budding region of the hydra than the other phosphatases (Chapter 3). On the basis of the present results, it may be hypothesized that both enzymes (alkaline phosphatase and to a lesser extent ATPase) play a role in growth processes. These findings are similar to those of Moog ('50, '51, '53, '59) who showed that undifferentiated tissue was relatively rich in alkaline phosphatase.

The findings reemphasize the results of other investigators obtained with other animals. Powell ('51) found that alkaline phosphatase activity develops in the nephridia and integument of the annelid Eisenia foetida as these tissues differentiated during regeneration. In the ascidian Symplegma viride stolonica, ATPase appears simultaneously with the regeneration of zooids from undifferentiated stolons (Jaeger and Barth, '48). Alkaline phosphatase activity rises in the limb bud during regeneration of the limb of Amblystoma (Karczmar and Berg, '51). Similarly, there is a high level of alkaline phosphatase in the blastema of the regenerating tail of Triturus (Ghiretti, '50). Acetylcholinesterase activity is low

during the early formative phases of development and then increases as regeneration proceeds in the forelimb of the adult newt, Triturus (Singer et al, '60). Dukiet and Niwelinski ('60) found that in the regenerating forelimbs of Triturus alpestris dedifferentiating tissues show a decrease in B-glucoronidase but that this activity is restored again during differentiation. Niwelinski ('60) also found in this animal that DPNH and TPNH diaphorases, SDH, and glucosan phosphorylase activities also decrease during dedifferentiation but reappear during differentiation. Many enzymes have been found to increase during regeneration of the liver: alkaline and acid phosphatase, ATPase (Fortak, '59), glutamic dehydrogenase and glutamicaspartic transaminase (Greenbaum et al, '54). Also, there is a marked decrease in activities of succinoxiasse, malic dehydrogenase, cytochrome reductase and oxalacetic systems following partial hypatectomy but these activities increase as regeneration proceeds (Novikoff and Potter, '48).

Toward the end of the regenerative period when the tentacles have formed, a marked change in the distribution and activity of the phosphatases occurs. At this point, referring back to the original hypothesis, the hydra receives information as to its anatomical completeness. Then enzymes in the cells of the tentacles and peduncle, especially nematocysts, that appear uniquely involved in particular functions are formed or increase their



activity prior to or synchronously with the development of functional capacity. This finding is actually a reiteration of the notion brought out clearly by Barron ('41), Herrman ('53), and Shen ('55) that the change in this period is concerned with the development of biochemical mechanisms through which physiological activity becomes possible rather than with marked alterations in structural elements through which they are made manifest. The nematocysts are an excellent example of the process. These structures in the intact hydra only gain their enzymatic specialization when they reach the tentacles and peduncle (Chapter 3) and only then are they functional. The same appears to be true of these structures in the regenerate.

A final item concerns the notion of metabolic gradients in the hydra, as recently reviewed by Burnett ('61). This concept should be viewed with some degree of caution since it depends on how one defines metabolic. If this word encompasses enzymatic in its meaning, then the peduncle and tentacles are regions of high degree of activity instead of low as Burnett considered. Furthermore, if we take the case of the oxidative enzymes, succinic dehydrogenase, and DPNH diaphorase, certainly important cogs in any metabolic scheme, then there is no gradient either for the intact hydra or for the regenerate since there is a fairly even distribution of these enzymes

throughout in both animals.

Summary

1. The changes in histochemical localization of enzymatic activity undergone by the peduncle and base during its regeneration into a complete animal are reported in this chapter.

2. Acid phosphatase activity, which in the normal peduncle and base is very intense, was found to decrease markedly in intensity, especially in epidermal cells, early in regeneration. Toward the end of regeneration this enzyme increased in activity and a normal distribution was attained after five days. Alkaline phosphatase, on the other hand, remained at a fairly high level throughout regeneration, showing only a slight decrease after one day of regeneration.

3. The special phosphatases, glucose-6-phosphatase, 5'-nucleotidase, and ATPase, intensely reactive in the normal peduncle and base, showed a striking loss of activity after one day of regeneration. These enzymes then increased in intensity of reaction as regeneration proceeded, ATPase reappearing sooner than the other enzymes, until the normal regional distribution of enzyme activities was attained after five days of regeneration.

4. DPNH diaphorase and SDH, in contrast to all other enzymes investigated, showed no changes in cytoplasmic localization or intensity of reaction during regeneration.

5. On the basis of these results, the regeneration of the peduncle and base into a mature hydra has been divided into three stages. In the first stage, that of enzyme de-differentiation, the cells of the peduncle and base lose their functional (enzymatic) specialization as they change from a normal situation to one in which rapid growth will become the major expression. During the second stage, that of active growth, the distribution of enzymatic activity in the regenerate was quite similar to that occurring in the growth and budding regions of intact hydra and in small lateral buds. This similarity may signify that growth regions in intact hydra or in regenerates have a different enzymatic pattern than the other areas of the intact hydra which have become specialized morphologically and functionally. Of particular interest is the absence of acid phosphatase containing bodies (lysosomes) during the growth phase. The disappearance of the bags of enzymes (lysosomes) may be taken as a sign that there is an overall decrease in catabolic activity during regeneration. The final stage of regeneration is that of enzyme differentiation in which specialized cells, especially nematocysts, of the tentacles, peduncle, and base, acquire enzymatic specialization simultaneously with the appearance of functional capacities.

6. It is hypothesized that the hydra receives information

as to its anatomical completeness and that lacking this information during regeneration the hydra concentrates on growth, all cells attaining an enzymatically unspecialized state. Regeneration proceeds until the hydra is anatomically complete at which time cells reattain their enzymatic and therefore functional specialization.

Explanation of Figures

Figs. 1-4 Acid phosphatase activity in regenerating hydra

Fig. 1 Peduncle and base immediately after excision demonstrating intense acid phosphatase activity especially in the distal area of the peduncle (p) and in the base (b).

Fig. 2 Peduncle and base stained for acid phosphatase after one day of regeneration. Note the striking loss of activity in epidermal cells and retention of activity in nematocysts.

Fig. 3 Three day regenerate stained for acid phosphatase. There is almost no activity in epidermal cells of all regions. The activity in the developing tentacles (t) is due to the intense capsular activity of mature nematocysts. A few scattered nematocysts in the remainder of the hydra are reactive.

Fig. 4 Hydra after four days of regeneration stained for acid phosphatase. The enzyme distribution of the mature hydra is partially attained at this time. The tentacles and peduncle are the most active regions due mainly to the activity in the maturing nematocysts. Note however, that some activity is appearing in epidermal cells.

Figs. 5,6 Glucose-6-phosphatase activity

Fig. 5 One day regenerate. Practically all enzyme activity is lost (compare with peduncle and base (b) in Fig. 6.

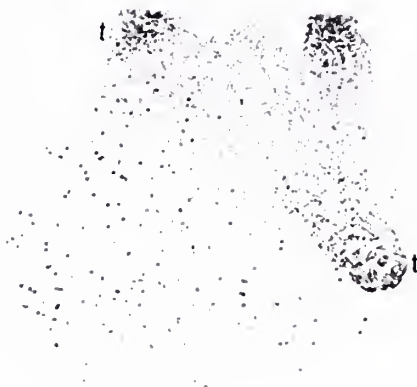
Fig. 6 Five day regenerate. The normal regional distribution of intense activity in the tentacles, peduncle, and base has returned at this time.



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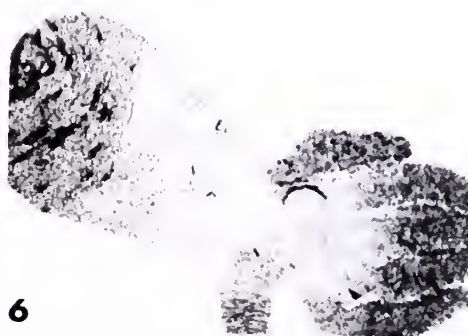
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Figs. 7-10 demonstrate the changes in alkaline phosphatase activity undergone by the regenerating peduncle and base.

Fig. 7 Peduncle and base stained for alkaline phosphatase immediately after excision. Intense activity is present in epidermal cells and capsules of nematocysts.

Fig. 8 One day regenerate stained for alkaline phosphatase. Note that the rapidly growing distal region shows some loss of activity while the cells of the base and nematocysts remain intensely stained.

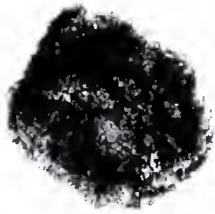
Fig. 9 Alkaline phosphatase activity in the three day regenerate. Note that intense activity is present in all regions except the base (b).

Fig. 10 Alkaline phosphatase activity in the four day regenerate. Activity of all areas is at a high level at this time.

Figs. 11-12 demonstrate DPNH diaphorase activity in the regenerating hydra.

Fig. 11 Peduncle and base stained for DPNH diaphorase activity immediately after excision. Note the even distribution of activity in all areas.

Fig. 12 Four day regenerate showing DPNH diaphorase activity. Note that the normal peduncle (Fig. 11) and the four day regenerate show no appreciable differences in intensity of reaction.



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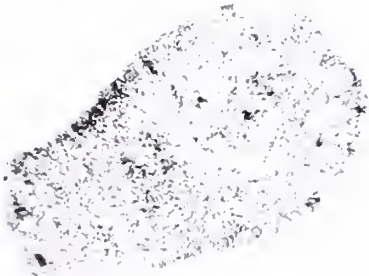


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Figs. 13-16 illustrate changes in intensity and localization of 5'-nucleotidase activity in the regenerating peduncle and base of hydra.

Fig. 13 Peduncle and base after removal stained for 5'-nucleotidase activity. Note the intense activity present in the peduncle (p); the base (b) is somewhat less reactive.

Fig. 14 One day regenerate showing 5'-nucleotidase activity. Note the remarkable loss of activity. Only cell membranes and a few associated granules are reactive at this stage.

Fig. 15 After three days of regeneration 5'-nucleotidase activity is present in the epidermal cells and nematocysts of the tentacles and is beginning to appear in the cells of the peduncle. A few scattered loci of cells and nematocysts are reactive in the remainder of the hydra.

Fig. 16 Hydra after four days of regeneration showing 5'-nucleotidase activity. Intense activity is now present in the epidermal cells and nematocysts of the tentacles and peduncle. The base is still unreactive at this stage.



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Figs. 17-20 demonstrate the changes in ATPase activity undergone by the regenerating peduncle and base of hydra.

Fig. 17 Peduncle and base stained for ATPase activity immediately after removal. Hematocysts and epidermal cells of the peduncle (p) are intensely reactive while the base (b) shows a somewhat less intense reaction.

Fig. 18 One regenerate stained for ATPase activity. Only some activity remains in the cells of the distal portion of the peduncle while the base remains intensely stained.

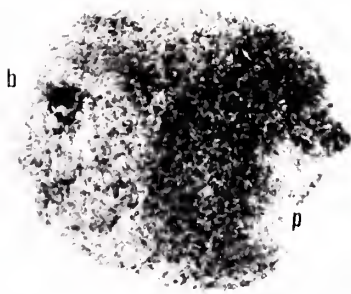
Fig. 19 Three day regenerate stained for ATPase activity. Many of the cells of the regenerate show moderate activity with the exception of those in the base which are unreactive at this stage.

Fig. 20 Hydra after five days of regeneration stained for ATPase activity. Much of the activity in the stomach has disappeared while the tentacles and peduncle (p) now show intense activity.

Figs. 21 & 22 illustrate SDH activity in the regenerating hydra.

Fig. 21 One day regenerate stained for SDH activity. Note the even distribution of enzyme activity.

Fig. 22 Four day regenerate stained for SDH activity. The enzyme is evenly distributed throughout all areas of the hydra and the intensity of reaction is identical to that in the one day regenerate (Fig. 21).



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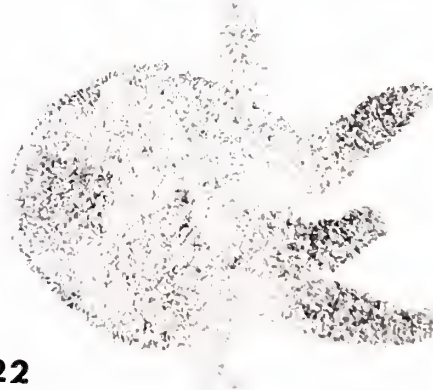
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Chapter 7

The Role of the Nervous System in the Regenerating Hydra

A study of the distribution and changes in enzyme activity of the regenerating hydra (Chapter 6) revealed three stages of regeneration. The first was a stage (occurring soon after severing the hydra below the budding region) of enzymatic dedifferentiation in which cells lost some of their enzymatic specialization associated with the mature, functional state. The second stage was one of active growth of regenerates accompanied by a different enzymatic set. The final stage was that of enzymatic differentiation in which cells of the regenerating hydra reacquired the enzymatic specialization of the mature state simultaneously with the appearance of functional capacities. Besides these interesting enzymatic changes associated with regeneration, the findings implied that the hydra possessed a system for the recognition and dissemination of biologic information since incompleteness was associated with stages one and two, while completeness of anatomical form appeared to be recognized and signaled the onset of stage three.

On these bases, it was felt that a nervous system in hydra, not observed or denied in some recent electron microscopic studies (Hess et al., '57; Chapman and Tilney, '59, '59a; Slautterback and Fawcett, '59; R. Wood, '59; Hess, '61), but supported by a preponderance of light

microscopic morphological studies (Korotneff, 1876; Jickeli, 1882; Schneider, 1890; Zoja, 1892; Wolff, '04; Hadzi, '09; Marshall, '23; McConnell, '32; Hyman, '40; Mueller, '50; Semal-van Gansen, '52; Spangenberg and Ham, '60), could serve to disseminate the necessary information concerning anatomical completeness or incompleteness. If the nervous system served to distribute this information and thereby regulate the processes of regeneration by neuronal or neurohumoral means, then inhibition of the nervous system should also inhibit regeneration. Therefore, to indirectly categorize some of the actions of the nervous system, which was studied histologically and histochemically during regeneration, the effects of neuropharmacological agents of regeneration of hydra was investigated.

Material and Methods

Neuropharmacological studies. Mature, well fed, Hydra littoralis, possessing 6 tentacles, grown in culture by the method of Loomis and Lenhoff ('56) as modified by Burnett ('59), were used as experimental animals. These were bisected through the stomach just below the growth region and allowed to regenerate without feeding. Most of the animals were placed in culture media containing small concentrations of neuropharmacological agents: physostigmine (eserine) ($2.4 \times 10^{-4} M$), hexamethonium ($3.7 \times 10^{-4} M$), diisopropylfluorophosphate (DFP) ($1 \times 10^{-5} M$),

atropine sulfate ($3 \times 10^{-4}M$), hemicholinium ($7 \times 10^{-4}M$), decamethonium ($4.6 \times 10^{-4}M$), d-tubocurarine ($2.9 \times 10^{-4}M$), reserpine ($1.6 \times 10^{-6}M$), xylocaine ($4.3 \times 10^{-4}M$), ergotamine tartrate ($7.7 \times 10^{-5}M$), amphetamine ($5.3 \times 10^{-4}M$) methyldopate (ethyl ester of levo-3-(3,4-dihydroxyphenyl)-2-methylalanine) ($3.3 \times 10^{-4}M$), and chlorpromazine ($3.5 \times 10^{-5}M$).

The final concentrations of pharmacological agents used in these experiments were selected as a result of preliminary experiments in which greater and lesser concentrations were used. The lesser concentration showed the effects to be reported less dramatically and much greater concentrations killed the hydra in the case of most of the neuropharmacological agents. Therefore, the dose used was considered a mean that would show an effect and still not be lethal. Not counting these pilot experiments, at least 25 hydra were used for each of the final experiments with each of the drugs. These various experiments were carried out for 5 to 20 days. The controls for these experiments were bisected hydra not subjected to pharmacological agents, or those bisected hydra subjected to neuropharmacological agents for 5 to 12 days before transfer to plain media alone. All animals were observed daily prior to their change of media and their size and shape were noted.

Since all neuropharmacological agents inhibited to some degree growth and differentiation, attempts were

hopefully made to reverse the effects of these drugs by adding some biologically important substances, thought or known to be endogenously depleted, to the incubating media. Thus, since ergotamine is an adrenergic blocking agent, epinephrine ($5.5 \times 10^{-5} \text{M}$) and norepinephrine ($5 \times 10^{-5} \text{M}$, $5 \times 10^{-6} \text{M}$) were placed in the media along with the inhibitor. Epinephrine ($5.5 \times 10^{-5} \text{M}$), norepinephrine ($5 \times 10^{-5} \text{M}$, $5 \times 10^{-6} \text{M}$), and 5-hydroxytryptamine (5-HT) ($1 \times 10^{-4} \text{M}$) were added along with reserpine and methyldopate. Since DFP, physostigmine, atropine, and tubocurarine interfere with cholinergic mechanisms, acetylcholine (ACh) ($2.1 \times 10^{-3} \text{M}$, $1 \times 10^{-3} \text{M}$) and acetyl-B-methylcholine ($2.6 \times 10^{-3} \text{M}$, $1.3 \times 10^{-4} \text{M}$) were added along with these inhibitors. The latter compound is cholinergic but is less rapidly hydrolyzed than acetylcholine. Regenerates were also placed in the above replacement drugs without any neuropharmacological inhibitors. Regenerates were observed between 36 and 72 hours of regeneration. Normal proximal halves of a bisected hydra begin to develop tentacles at about 36 hours while the drug inhibited hydra do not. If any reversal of inhibition occurred it could be most readily noted at this time by the appearance of at least tentacle buds.

Histological studies. Whole mounts of regenerates were studied in order to determine some of the cellular effects of the neuropharmacological agents. Regenerates were placed in a solution of 0.5% methylene blue reduced by

sodium formaldehyde sulfoxalate as described by McConnell ('32) and observed after 2, 6, 12, 24, and 36 hours of regeneration. The number of nerve cells at the regenerating surface of proximal and distal sections of hydra bisected below the growth region were counted in control regenerates and those subjected to inhibitory drugs.

In addition, the effects of drugs on interstitial cells were indirectly observed by counting cnidoblasts at the cut surface, since the latter are easily recognized in whole mounts and normally differentiate rapidly from interstitial cells. Presumably, an increase or decrease in the number of interstitial cells would be reflected by corresponding changes in numbers of cnidoblasts. The results reported represent an average of ten experiments.

Histochemical studies. Since the distribution of some enzymatic activities in the normal hydra had already been categorized (Chapter 3) as well as the changes in enzyme patterns that accompany normal regeneration (Chapter 6), some enzyme histochemical studies (adenosine-5-phosphatase (AMPase), acetylcholinesterase (AChE), and acid phosphatase) were conducted to compare the distribution of enzymes in both normal regenerates and those subjected to neuropharmacological agents. In addition, the localizations of AChE, epinephrine, norepinephrine, 5-HT, and monoamine oxidase (MAO) (Glenner et al., '57) were studied in the nervous system of 36 hour control and drug adminis-

tered regenerates, since many of the neuropharmacological agents employed inhibit or deplete these substances or activities. Most of these histochemical methods applied to hydra have been described previously (Chapter 3 and 4).

Observations

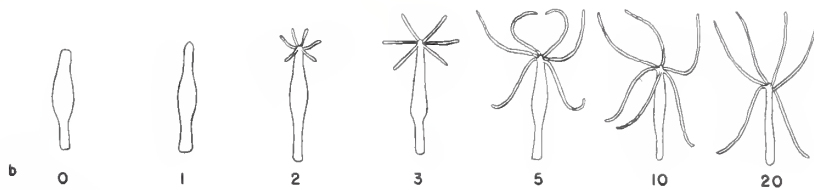
The time sequence and changes in form and shape during normal regeneration of hydra are presented as a background before considering the results of the experiments in which pharmacological agents were used. The body of the distal half (containing the tentacles) of the bisected hydra increased in length for 5 or 6 days and a recognizable peduncle and base were present after 7 days (Fig. 1a). The proximal half (stomach, budding region, peduncle, and base) of the hydra began to develop a hypostome and tentacle buds after 36 hours of regeneration. The body of the hydra lengthened and the tentacles grew until normal form was attained after 4 to 5 days (Fig. 1b).

Effects of neuropharmacological agents. The tentacles of the distal half of the bisected hydra contracted and assumed a knobby appearance after one day in solution of media containing physostigmine ($2.4 \times 10^{-4}M$). At day 2, the distal portion appeared to be unchanged, but after 3 days, the tentacles appeared only as small protuberances from the body of the hydra which was smaller in size than that of the controls. During the 4th and 5th days, the hydra assumed a star-shaped appearance, and all vestiges

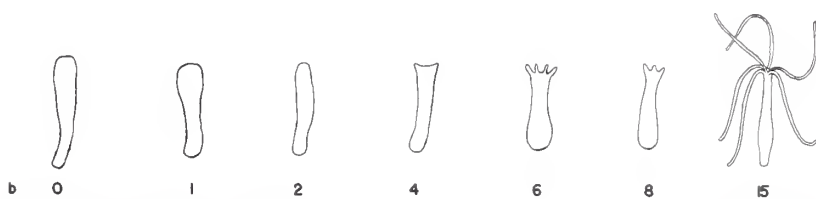
Text fig. 1 Normal regeneration of bisected hydra. Note that tentacles have appeared in the proximal portions (b) by day 2. The proximal portion acquired normal form at day 5 while the distal half (a) required an average of 7 days to develop a base.

Text fig. 2 Hydra regenerating in physostigmine (eserine) ($2.4 \times 10^{-4} M$). The distal portion (a) assumed a star shape at day 6 and tentacles disappeared by 8 days. Note that tentacle buds appeared at day 6 in the proximal half (b) but never assumed any length. Transfer of both portions to plain water without inhibitor at day 8 resulted in acquisition of normal form by day 15.

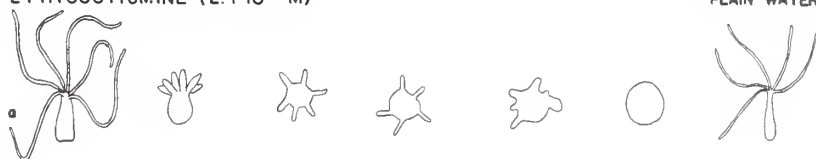
Text fig. 3 hexamethonium ($3.7 \times 10^{-4} M$) treatment produced a star shaped form at day 2 in the case of the distal portions (a). Proximal halves (b) demonstrated aborted tentacle formation at day 8 but these disappeared to produce an oblong mass of cells. The distal portion (a) similarly assumed a round shape by day 12. Transfer of the round masses to plain water at this time resulted in recovery, regeneration, and assumption of normal form by day 19.



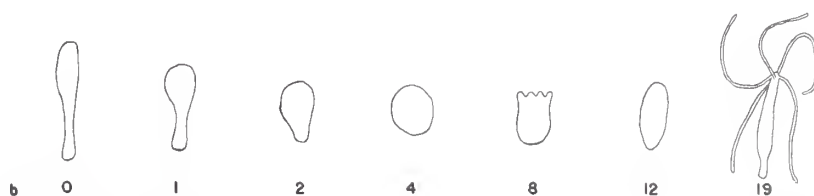
1 NORMAL



2 PHYSOSTIGMINE ($2.4 \cdot 10^{-4} M$)



PLAIN WATER



3 HEXAMETHONIUM ($3.7 \cdot 10^{-4} M$)

PLAIN WATER

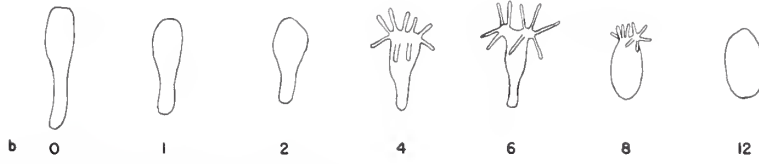
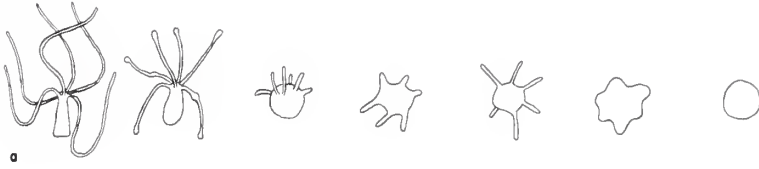
of the tentacles disappeared at the 6th or 7th day (Fig. 2a). Following this stage, the cells of the remaining round mass began to dissociate, an effect accomplished by the peeling off of living cells from the periphery of the mass. The proximal portion, on the other hand, assumed an oblong shape by the first day. About 20% of the regenerates began to develop tentacle buds at day 4 and 50% at day 6 (Fig. 2b). The tentacles, however, never assumed any length and disappeared after 9 days. The remaining mass of living hydra cells dissociated after this. Transfer of the inhibited regenerates to media without eserine at any time of the experiment resulted in resumption of normal regeneration and eventual attainment of normal form.

Hexamethonium ($3.7 \times 10^{-4} M$) caused a marked contraction of regenerating parts and diminution in the size of the tentacles of the distal portion was noted after one day. A bizarre stellate form was maintained for several days and by the 10th day, the regenerate assumed a round shape which was sustained for approximately 4 more days before signs of dissociation, described above, were noted (Fig. 3a). The proximal portion also became a round cellular mass by the 3rd day. In some of these regenerates, very small tentacle buds appeared on day 4 to 8 but disappeared thereafter (Fig. 3b). The resulting small mass also dissociated after 14 days. Transfer of the small cellular masses to fresh water resulted in development of

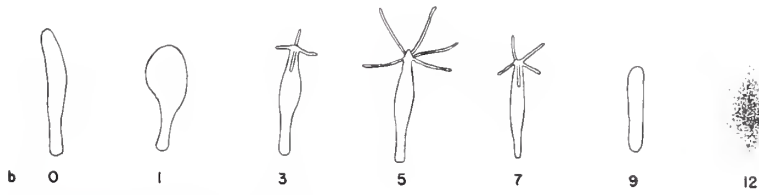
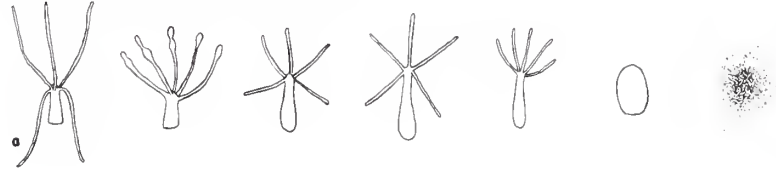
Text fig. 4 Decamethonium ($4.6 \times 10^{-4} M$) resulted in the production of a stellate form at day 4 in the case of the distal half (a). The tentacles disappeared by day 8 and a round mass of cells remained at day 12. The proximal half (b) developed stunted tentacles by day 4. In this case, two heads were formed after which the tentacles regressed to leave an oblong mass at day 12 which dissociated thereafter.

Text fig. 5 Tubocurarine ($2.9 \times 10^{-4} M$) resulted in the appearance of bulb-shaped enlargements on the tentacles of the distal half (a) at day 1. The tentacles regressed to leave a round mass (day 9) which dissociated by day 12. The proximal portion (b) developed short tentacles between the 3rd and 7th days which disappeared at day 9. The resulting mass of cells dissociated at day 12. However, if they were transferred to water before day 12, normal regeneration took place.

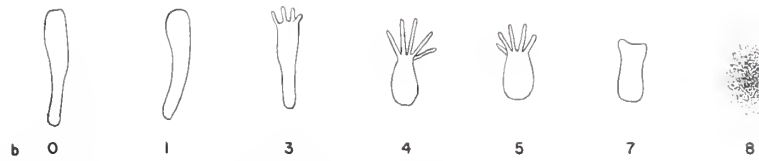
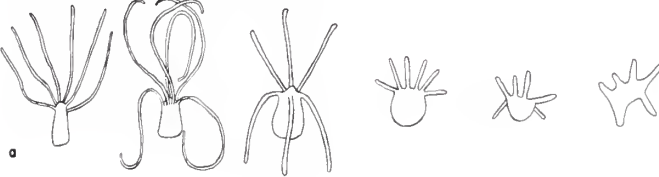
Text fig. 6 Regeneration in DFP ($1.0 \times 10^{-5} M$) resulted in the production of a star-shaped form at day 5 in the case of the distal half (a) which dissociated at day 8. The proximal portion (b) showed limited tentacle formation at day 3 and dissociation at day 8. These effects of DFP were irreversible.



4 DECAMETHONIUM ($4.6 \cdot 10^{-4} M$)



5 TUBOCURARINE ($2.9 \cdot 10^{-4} M$)



6 DIISOPROPYLFLUOROPHOSPHATE ($1 \cdot 10^{-5} M$)

tentacles and assumption of normal form in approximately 7 days.

Decamethonium ($4.6 \times 10^{-4} M$) treatment resulted in a shortening of the tentacles in the distal half by the first day and production of a stellate form by 3 days. These portions thereafter became round and began to dissociate at day 12 (Fig. 4a). The proximal portion developed stunted tentacles after 3 days in decamethonium. These animals remained in a stunted form and usually dissociated after about two weeks. About 25% of the proximal halves developed two heads and one animal grew a tentacle from the base (Fig. 4b). Transfer of the portions to fresh water did not result in resumption of normal regeneration; instead, they continued to regress eventually disintegrating completely.

Tubocurarine ($2.9 \times 10^{-4} M$) treatment resulted in the appearance of bulb-shaped enlargements on the tentacles of the distal half at day 1. These disappeared during the second day and regression of the tentacles was noted. After about 7 days, the regenerate decreased in size, became round, and dissociated after 12 days in tubocurarine (Fig. 5a). The appearance of tentacles in the proximal half of the regenerate was delayed until the third day, after which the tentacles disappeared and the cell mass dissociated at about day 12 (Fig. 5b). Two regenerates grew tentacles from the body. Regeneration

proceeded normally when hydra were transferred to fresh water prior to dissociation.

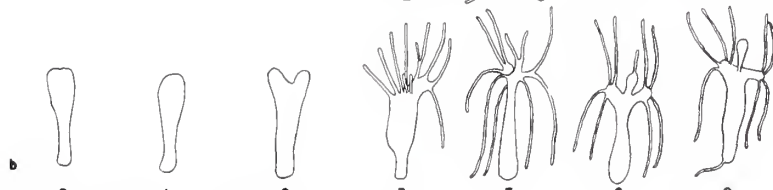
DFP ($1 \times 10^{-5} \text{M}$) caused an initial relaxation of the distal half of the hydra and the tentacles became quite long and wavy. However, the tentacles began to shorten by the 3rd or 4th day, disappearing in some of the hydra by 5 days. The hydra then assumed a bizarre stellate or a round form which was maintained until it dissociated after 8 days (Fig. 6a). The proximal portion of the hydra developed tentacle buds at the 3rd to 5th days, but these disappeared by day 7 and the round mass of cells began to dissociate at the 8th or 9th day (Fig. 6b). The effects of DFP were irreversible as transfer to plain media did not result in regeneration.

Hemicholinium ($7 \times 10^{-4} \text{M}$) did not appear to affect regeneration of the distal half for the first 8 days (Fig. 7a) but thereafter the hydra became stellate in shape by day 12 and began to dissociate on the 14th day. The proximal half did not develop tentacle buds until the third day and contrary to the events in the distal half, a normal form was attained at day 10 in some of the hydra. However, 20% of the proximal portions developed two heads and two proximal regenerates possessed one tentacle arising from the base in addition to the tentacles occurring at the distal end. Another 25% possessed forked tentacles (Fig. 7b). Transfer of these animals on day

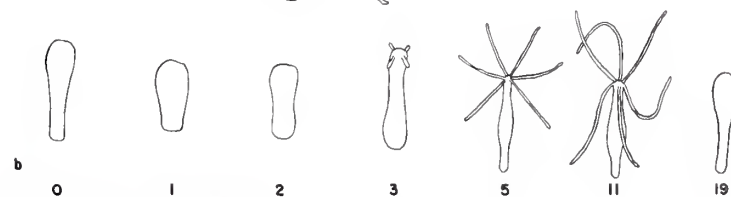
Text fig. 7 Distal portions (a) regenerating in hemicholinium ($7.0 \times 10^{-4} M$) were normal until about day 8 when regression with eventual dissociation began. The proximal half (b) frequently developed into bizarre shapes. In the case illustrated two heads appeared at day 3. Two tentacles protruding from a stump of tissue were evident at day 5. A tentacle grew from the base at day 8. Both halves regressed thereafter unless placed in fresh water and this was accompanied by normal regeneration including reorganization of the proximal part.

Text fig. 8 Atropine ($3.0 \times 10^{-4} M$) treatment produced the unusual star shape at day 11 which became round at day 19 in the case of the distal half (a). Tentacle formation was delayed in the proximal portion (b) until day 3 and regression was apparent at day 19.

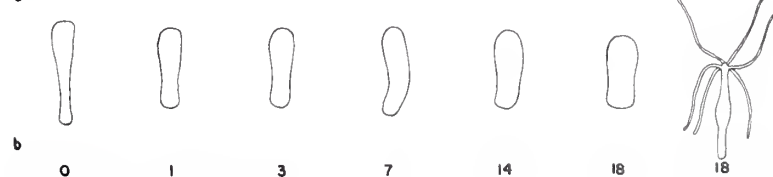
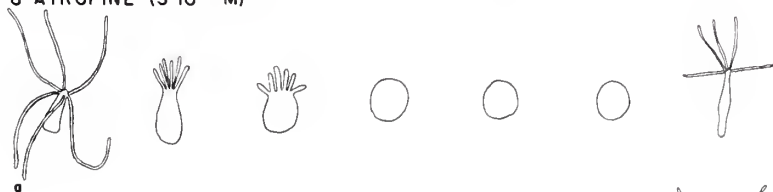
Text fig. 9 Reserpine ($1.6 \times 10^{-6} M$) treatment resulted in the assumption of round masses of cells which could be maintained for three weeks in this form. Transfer without reserpine at day 14 resulted in normal regeneration complete after 4 more days.



7 HEMICHOLINIUM ($7 \cdot 10^{-4}$ M)



8 ATROPINE ($3 \cdot 10^{-4}$ M)



9 RESERPINE ($1.6 \cdot 10^{-6}$ M)

PLAIN WATER

8 to 12 to fresh water resulted in reorganization and attainment of normal form in 5 to 7 days.

Atropine ($3 \times 10^{-4} \text{M}$) apparently did not affect the regeneration of the distal portion until the 4th or 5th day when the tentacles decreased in size. The hydra then assumed an unusual star shape (Fig. 8a). This drug retarded the development of tentacles in the proximal half for three days. However, regeneration then proceeded normally until about the 14th day when the regenerates began to regress, losing their tentacles and assuming a round shape (Fig. 8b). Transfer to fresh water resulted in assumption of normal form.

Reserpine was one of the most potent substances tested, being effective at a concentration of $1.6 \times 10^{-6} \text{M}$ (about 1:1,000,000). The tentacles of the distal portion shortened at day 2 and disappeared at day 4 (Fig. 9a). The proximal portion became round in shape at day 2 or 3 without appearance of tentacles (Fig. 9b). These round cell masses, both from proximal and distal regenerates, could be maintained in this form for over three weeks until they dissociated. Transfer of the inhibited portions to fresh water resulted in rapid recovery and normal regeneration.

Portions placed in xylocaine ($4.3 \times 10^{-4} \text{M}$) regenerated normally but at a slower rate. Proximal halves did not develop tentacle buds until $2\frac{1}{2}$ days and achieved

normal form at 7 days. Distal halves did not develop a base until 13 days.

Ergotamine ($7.7 \times 10^{-5} \text{M}$) only delayed the appearance of tentacle buds on the proximal portion for 12 hours beyond the normal controls. About half of the distal portions did not develop normally, but became stellate by 2 or 3 days. This effect was not reversible by placing the regenerates in fresh water.

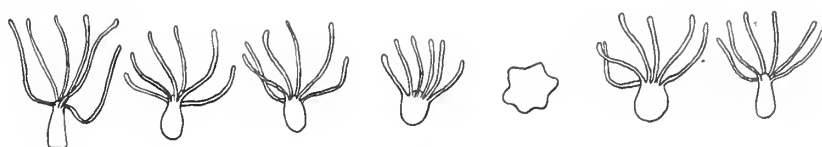
Portions placed in methyldopate ($3.3 \times 10^{-4} \text{M}$) regenerated normally until the fifth day when regression became apparent. Both portions assumed a round shape by day 7 and dissociated the next day. Transfer to fresh water did not reverse this process in most cases although approximately 25% did eventually regain normal form after two weeks (Fig. 10a,b).

Amphetamine ($5.3 \times 10^{-4} \text{M}$) delayed the development of tentacle buds in the proximal half for about 12 hours. Regeneration then proceeded normally until day 5 when the tentacles shortened and the regenerate became round. Most of these masses dissociated by day 7 but a few recovered when placed in fresh water (Fig. 11b). The distal portions, on the other hand, assumed forms consisting of short tentacles protruding from a central mass of cells. Surprisingly, these forms decreased in size until they were about $\frac{1}{2}$ mm in diameter and resembled tiny stars (Fig. 11a). Transfer to fresh water did not

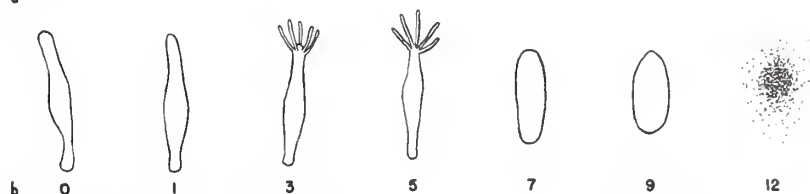
Text fig. 10 Portions placed in methyldopate ($3.3 \times 10^{-4} M$) regenerated normally until the fifth day when regression became apparent. In this case, transfer to fresh water resulted in recovery and regeneration of the distal (a) but not of the proximal half (b).

Text fig. 11 Amphetamine ($5.3 \times 10^{-4} M$) produced unusual star-shapes in the case of the distal half (a) which decreased in size until they were about $\frac{1}{2} mm$ in diameter at day 14. Tentacle formation was delayed slightly in the proximal half (b) followed by regression and dissociation at day 14.

Text fig. 12 Chlorpromazine ($3.5 \times 10^{-5} M$) treatment of the distal portion (a) caused regression at day 4, loss of form at day 5, and dissociation at day 6. The proximal portions (b) did not develop tentacles and dissociated at day 6.



a



b

0

1

3

5

7

9

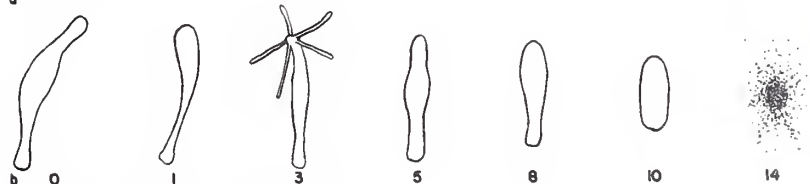
12

10 METHYLDOPATE ($3.3 \cdot 10^{-4} M$)

PLAIN WATER



d



b

0

1

3

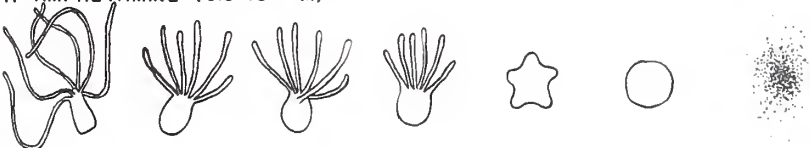
5

8

10

14

11 AMPHETAMINE ($5.3 \cdot 10^{-4} M$)



a



b

6

1

2

3

4

5

6

12 CHLORPROMAZINE ($3.5 \cdot 10^{-5} M$)

prevent or reverse this process.

Chlorpromazine ($3.5 \times 10^{-5}M$) had marked effects on regenerating hydra. The proximal portions were unable to develop tentacles and dissociated by day 4 or 5 (Fig. 12b). The distal half regressed to a stellate form at day 3 or 4, became round, and dissociated at day 5 or 6 (Fig. 12a). Transfer to fresh water resulted in recovery and normal regeneration in about half of the regenerates.

Efforts to reverse the effects of the neuropharmacological drugs by adding biologically active amines and other compounds were unsuccessful. In all cases, tentacle buds did not appear at the normal time but at the same time as those of regenerates subjected to the inhibitor neuropharmacological agents alone. However, the reversal compounds were not totally without effect. Contrary to the hope of reversal, some of these compounds appeared to augment some of the effects of the neuropharmacological agents, since there was an increased number of abnormal forms of regenerating hydra placed in solution containing inhibitor plus replacement drug than those subjected to inhibitor alone. This was most marked in the case of eserine plus methylcholine where 50% of the regenerates developed two heads while those in eserine or acetyl-B-methylcholine alone did not show this change. In one case, a regenerate in methyldopate and norepinephrine rapidly developed a long tentacle protruding

from the peduncle. Regeneration proceeded normally when severed hydra were placed in solutions of the replacement drugs alone.

Histological studies. Nerve cells and fibers are clearly demonstrated by the methylene blue technique of McConnell ('32) (Figs. 14, 15). The nervous system of normal, regenerating and sexual hydra is being studied in detail by Burnett (personal communication) with this method. The nervous system of hydra underwent profound changes during regeneration (Table 1, Fig. 13). Normally, there were on the average of 20 stained nerve cells at the cut surfaces immediately after severing the hydra (Fig. 16). However, after 2 hours there was a slight decrease in the number of nerve cells at the regenerating surface (Fig. 17). At six hours, the number of nerve cells increased slightly (Fig. 18). A large increase in number then occurred, reaching a peak at 12 hours, to produce more than twice the number of nerve cells observed immediately after transection (Figs. 19, 20). Following this increase, there was a gradual decline until at 36 hours only a few nerve cells were present at the regenerating surface. This time coincided with the beginning development of tentacles in the proximal regenerate.

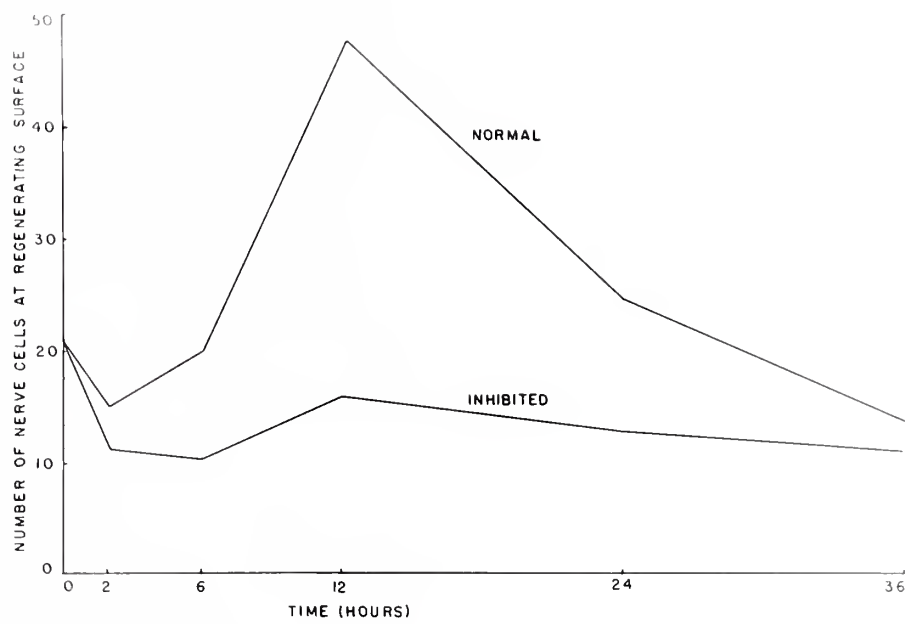
A strikingly different result was obtained in the neuropharmacologically treated regenerates (Table 1, Fig. 13). Although the early changes were similar to those

TABLE 1

Effect of neuropharmacological agents on number of neurons and cnidoblasts at the regenerating surface of distal (D) and proximal (P) sections of hydra bisected below the growth region. Each figure represents the average of 10 experiments.

Time (Hours)	Neurons						Cnidoblasts	
	0	2	6	12	24	36	0	12
Controls D	20	14	17	46	24	10	15	29
P	21	15	20	48	25	12	16	30
Eserine D	15	17		13	13	12		24
P	16	12		16	15	16		30
Hexamethonium D	8	3		8	21	9		20
P	12	8		11	21	7		21
DFP D	9	11		15	5	7		29
P	12	7		13	18	10		25
Tubocurarine D	10	12		20	12	6		25
P	12	8		15	7	11		32
Atropine D	13	10		13	14	15		34
P	14	8		12	9	9		28
Reserpine D	9	9		13	5	6		31
P	8	10		12	9	10		35
Ergotamine D	8	14		22	9	9		27
P	8	13		17	24	16		32
Methyldopate D	8	10		14	13	10		19
P	11	10		17	7	12		21
Amphetamine D	6	9		18	8	8		28
P	13	14		20	12	12		25
Chlorpromazine D	9	14		11	5	5		25
P	9	15		12	9	5		28

Text fig. 13 Graph of the number of nerve cells at the cut surface of the proximal half of a transected hydra at various times (0-36 hours) after transection. The normal curve (upper) represents the average of the findings on 20 animals. The bottom curve was constructed from neuron counts on 100 transected hydra subjected to a variety of inhibitory neuropharmacological agents (10 animals for each inhibitory drug). The data for the individual drugs are presented in Table 1. The graph demonstrates that in the presence of neuropharmacological inhibitors, the normal increase in number of nerve cells at the regenerating surface does not occur.



observed in the control preparations, the large increase in nerve cells at 12 hours did not occur in the presence of the drugs. Instead, there was only a small increase between 12 to 24 hours over that present at two hours (Figs. 21 - 25).

Normally, cnidoblasts doubled in population at the cut surface by 12 hours of regeneration. With the exception of hexamethonium and methyldopate, the drugs did not appreciably affect the number of cnidoblasts found and thus presumably did not affect interstitial cells. In the cases of the two drugs mentioned, there was a moderate decrease in number respectively (Table 1).

Histochemical studies. The enzyme localizations of acid phosphatase, 5'-nucleotidase, and acetylcholinesterase were studied in regenerating hydra subjected to some of the neuropharmacological agents for 6 days. Normally, regeneration is completed after 4 or 5 days and the distribution of enzyme activities of that of the mature animal is attained. In the normal animal, cnidoblasts and epidermal cells of the peduncle, base, and tentacles show intense activity of several phosphatases while those in the hypostome, stomach, and budding region showed much less or no activity. AChE is present within neurons (ganglion and sensory cells) and neurites of the nervous system. These cells are slightly more numerous in the hypostome and base and some epidermal cells and cnidoblasts

of all regions also contain AChE activity (Chapter 3).

Contrary to these enzymatic situations in the normal regenerate those subjected to neuropharmacological inhibitors showed either a decrease in time of appearance of enzyme activity and/or a reduction in the numbers of active sites. For example, in both the distal and proximal portions subjected to eserine, acid phosphatase was localized only to a few loci of epidermal cells. Cells in the vestiges of tentacles were unreactive. AMPase activity was almost completely absent in all cells of both portions (Fig. 26). Similarly, AChE activity was markedly diminished in comparison to the normal localization. The latter finding may be due to the inhibitory action of eserine on this enzyme.

In the regenerates subjected to hexamethonium, decamethonium, and hemicholinium similar results were obtained concerning the distribution and sites of enzyme activity. In both the distal and proximal portions of hydra subjected to these drugs, acid phosphatase and AMPase were restricted to a few nematocysts primarily in the tentacles or tentacle buds (Figs. 26 - 29). Although there were significantly fewer active cells at 6 days in the treated hydra than the controls had after 4 days, it should be noted that the effect of hemicholinium was intermediate between the controls and those of the other drugs. In the developing peduncle and base

of the treated animals no phosphatase activity occurred and this finding was contrary to the control situation (Figs. 26, 28). In those regenerates from the distal portion, AChE was intensely active in epidermal cells and neuronal elements after hexamethonium and decamethonium, while after hemicholinium, a reaction of normal intensity occurred. In the regenerates from the proximal portion, intense AChE activity occurred only after decamethonium.

Nerve cells, in particular, were examined after 36 hours of regeneration in some specimens. Normally, in the 36 hour regenerates, nerve cells were reactive for AChE (Fig. 30), epinephrine, norepinephrine, 5-HT, and MAO. DFP (Fig. 31) and eserine abolished AChE activity within nerve cells and fibers. In the presence of ergotamine, epinephrine and norepinephrine staining was markedly reduced and epinephrine, norepinephrine, and 5-HT were completely absent in hydra treated with methyl-dopate. Norepinephrine and 5-HT staining was reduced slightly by reserpine. There was no MAO activity in animals regenerating in amphetamine.

Discussion

A variety of inhibitory neuropharmacological agents variably retard to various degrees or inhibit regeneration of hydra. Although these reagents could effect diverse and important factors in hydra cells (e.g., one or another metabolic activity or enzymatic activities associated with

vital functions other than metabolic) one factor was common; they all are known to cause some functional derangement of nervous tissue. DFP irreversibly inhibits cholinesterases (and is a potent inhibitor of other carboxylic acid esterases and other enzyme activities); eserine also inhibits cholinesterases, but reversibly; decamethonium is a depolarizing agent, but it exerts an inhibitory influence after the initial depolarization; tubocurarine, atropine, and hexamethonium block either pre- or post-ganglionic transmission or indeed in the case of the latter compound, ganglion cell transmission is blocked. Hemicholinium inhibits acetylcholine synthesis by blocking the incorporation of choline into the choline acetylase system, while reserpine depletes the content of catecholamines and 5-HT and prohibits their resynthesis. The remaining neuropharmacological drugs used also have different actions. xylocaine is a local anesthetic and blocks nerve transmission, while ergotamine is an adrenergic blocking agent. Amphetamine inhibits MAO activity and methyldopate blocks dopa decarboxylase activity and thus the synthesis of catecholamines and of 5-HT. Finally, chlorpromazine is a central nervous system depressant and tranquilizer possibly affecting respiratory enzymes (McIlwain, '62) and mitochondrial membranes (Spirtes and Guth, '61).

Therefore, while these compounds have widely

different modes of action and their effect may be simply to prevent the movements of cells in the regenerate, the fact that they all inhibit the functions of nervous tissues and regeneration simultaneously seems more than coincidental. On this basis it is suggested that the nervous system of hydra play a singular role in the processes of regeneration, growth, and differentiation. It should be recalled that neuropharmacological agents have been shown to inhibit regeneration in planaria (Welsh, '46, '48; Adamson et al., '62) and in amphibia (Singer et al., '55, Singer, '60). These effects were similar in result to those of the severing of nerves in these forms but in the present experiments drug action was a more expedient way of testing our hypothesis.

At this point, the existence of nervous tissue in the hydra should be re-emphasized. There is a preponderance of evidence from classical studies (see introduction), recent neurophysiological (Passano and McCollough, '62; Rushforth et al., '63), and histochemical studies (Chapters 3 and 4) that outweighs recent negative suggestions of "no nerves in hydra" (see introduction). Indeed, in Chapter 9 the fine structural details of hydra ganglion and sensory cells, neurites, and neurosecretory material are characterized. Similar findings were also reported for the jellyfish (MacKay, '63). More than that, it has been recently found that the hydra as

well as the sea anemone contains epinephrine, norepinephrine, and 5-HT (Chapter 4). On these bases it appears relatively certain that the nervous system of hydra exists. What was rather surprising was that this system functioned in the release of nematocysts (Chapter 5) and in the present work was associated with regeneration.

While the neuropharmacological agents inhibited, retarded, or confused the process of regeneration, presumably through inhibition of the nervous system, attempts to reverse this process by specific drug therapy (e.g. norepinephrine and 5-HT in the case of reserpine inhibition) were unsuccessful. It had been hoped that by these substitution experiments a more direct demonstration of the effect of the nervous system could be accomplished and incidentally these might indicate which of the potential neurohumors affected growth and differentiation. On reconsideration, however, the failure of nervous tissues in the framework of the present experiments and the imbalance in function caused by a variety of neuropharmacological drugs that act in different ways must be looked upon as extremely complicated and the substitution of one or another agents (ACh, catecholamines, or 5-HT) in retrospect would not be expected to completely reverse the inhibition of regeneration. This type of shot-gun attempt at therapy either accomplished nothing for the hydra or confused the process of regeneration even more.

This latter fact alone suggests some sort of action of some of the replacement drugs and the present experiments may have provided an incorrect dose at an inappropriate place and at the wrong time.

However, direct implication of the effect of neuropharmacological agents on nervous tissue was obtained by histological examination of stained nerve cells. Normally nerve cells double their number in 12 hours at cut surfaces of transected hydra, but this did not occur in regenerates subject to inhibitory agents. This finding alone suggests that one of the earliest events in regeneration involves the nervous system and in its absence, regeneration does not occur. In addition, it should be noted that little or no change in the number of cnidoblasts (and presumably of interstitial cells) occurred as a result of treatment with neuropharmacological agents and this may indicate the degree of specificity of the compounds used on nervous tissue.

The present work, in part, reiterates in a more primitive animal the finding of numerous authors on the effect of nervous tissue on the regeneration of higher forms. The dependence of regeneration on the nervous system was first noted by Todd (1823) in the aquatic salamander. Since then, the nervous system has been found to be necessary for the regeneration of many animals (see reviews by Korschelt, '27; Millet, '31;

Abeloos, '32). Some studies have been confined to invertebrates including arthropods (Needham, '45, '46), annelids (Morgan, '02; Holmes, '31; Zhinkin, '36; Salyles, '42). and platyhelminthes (Child, '04, '04a, '10; Welsh, '46, '48; Adamson et al., '62). In vertebrates, innervation has been found to be necessary for the regeneration of the extremities of the fish and lizard (Kamrin and Singer, '55, '55a) and larval (Schotté and Butler, '41, '44; Schotté and Harland, '43) or mature amphibians (Rose, '48; Singer and Craven, '48; Singer, '52, '59). Without detailed reviews of these works in which it was felt that nerves may serve as a pathway for cellular migration to the regenerating part (Needham, '52), may contribute cells to the formation of the regenerate (Guyenot and Schotté, '26; Thornton, '38; Chalkley, '54), may extend an influence through the epidermis of the regenerate (Singer, '49; Thornton, '54, '56), it is most pertinent to the present study that some authors considered the possibility that nerves may release a chemical substance necessary for regeneration and growth (Schotté, '26; Welsh, '46, '48; Needham, '52; Taban, '55; Singer, '52, '59, '60; Singer et al., '55).

In the present work, transected hydra, whose nervous system had been blocked by neuropharmacological agents, healed the wound by outgrowth of epithelio-muscular cells over the cut surface. Beyond this, there was retarded

or confused regeneration, complete inhibition, or regression depending on the concentration and type of the substance used. The regenerate hydra assumed a round or stellate shape and could be maintained in this form until their endogenous food supplies were exhausted. Normal form was not attained although aborted attempts at tentacle formation, resulting in bizarre forms, frequently occurred. Transfer of stunted hydra or those with abnormal shapes to fresh media resulted in the usual, though delayed pattern of regeneration except in the cases (e.g., DFP, decamethonium, ergotamine) where the effects of the drugs were irreversible. Distribution and amount of some enzyme activities occurring in normal regeneration (Chapter 6) were seriously modified and these activities were not reacquired so long as regeneration remained inhibited. This finding is another indication that differentiation had not occurred since the attainment of the enzymatic activities is associated with the mature functional state (Chapters 3 and 5). In addition, catecholamines and 5-HT were depleted in the several cases of inhibition for which they were tested, probably through a direct action of the treatment (e.g., reserpine). Thus, probable inhibition of the nervous system by drugs with different pathways of action maintains the regenerating hydra in a formless, undifferentiated state. This finding again strongly suggests that

the nervous system of hydra regulates the differentiation and growth of specialized cells in the hydra, the determination of polarity, and the attainment of normal anatomical form of the animal.

Coupling the finding of inhibition of regeneration by inhibition of nervous influences with the appealing suggestion, referred to in an earlier paragraph, of chemical transmitters, it may be worthwhile to examine the present findings according to the criteria set forth by Giarman ('59) for substances suspected of playing a role as chemical transmitters. Of course, it should be borne in mind that these substances would include neurohumors whether or not they affected transmission. This data is collected in Table 2.

Thus, according to these criteria there is suggestive evidence for the existence of chemical mediators in the nervous system of hydra. When the normal physiology of the nerve cell is altered by pharmacological agents which may interfere with chemical transmission, regeneration is retarded or completely blocked. If biologic information is recognized by nervous tissue and is disseminated to all cells of the body, interruption of any part of the pathway blocks the final effect. Thus, one component of the pathway of information could be chemical transmission of impulses within the neurite or at effector junctions. A distinct possibility in this chain may be

TABLE 2

Criteria which should be met by any naturally occurring substance suspected of playing a role in humoral transmission (Giarman, '59), as applied to the nervous system of the hydra.

1. Substances should be present in a discrete pattern in the nervous system.

Chemical techniques

5-hydroxytryptamine (Welsh, '60)

Histochemical techniques

Epinephrine

Norepinephrine

5-hydroxytryptamine

)
) (Chapter 4)
)

2. Enzymatic mechanisms for the synthesis and destruction of these compounds should be present.

Chemical techniques

Acetylcholinesterase (Mitropolitanskaya, '41)

Histochemical techniques

Acetylcholinesterase (Chapter 3)

Monoamine oxidase (present chapter)

3. Increase or decrease in the concentration of these compounds should result in demonstrable effects.

Acetylcholine

Epinephrine

Norepinephrine

5-hydroxytryptamine

Histamine

)
) Augment nematocyst
) discharge (Chapter 5)
)
)

Decrease in epinephrine, norepinephrine, and 5-HT by neuropharmacological agents coincides with inhibition of regeneration (present chapter).

4. Blocking agents of the substances or enzymes should produce demonstrable effects.

Hexamethonium

Tubocurarine

) Inhibit effects of acetyl-
) choline on nematocyst dis-
charge (Chapter 5)

Atropine

Tubocurarine

Hexamethonium

Physostigmine

Diisopropylfluorophosphate

Hemicholinium

Decamethonium

Ergotamine

Methyldopate

)
)
)
) Inhibit regeneration
) (present chapter)
)
)
)

the release of neurohumors by neurite endings. Another interpretation consistent with the present data is that biologic information of one sort or another is recognized by all cells. However, in this circumstance a trophic factor elaborated by nervous tissue would be required for expression. As before, interference with nervous function would interfere in the chain of events that culminated as an expression of biological information - in the present instance, the events associated with normal regeneration.

A final matter to be discussed is the hypostomal growth factors first enunciated by Burnett ('61, '62). This investigator hypothesized as a result of grafting experiments that a principle in the hypostome stimulates cell proliferation in interstitial cells, digestive cells, and epitheliomuscular cells and is responsible for the continual growth of the body column. In addition, an inhibitory principle was thought to be produced in the growth and budding regions which inhibits cellular divisions in the gastric region and peduncle. According to Burnett, the inhibitory principle, which might be a metabolic by-product, is produced in areas (growth and budding regions) where metabolism is supposedly high and cell divisions are frequent.

On the basis of the present work, this theory may be partially correct in principle but incorrect in detail.

The present studies indicate that the nervous system of hydra controls not only the maintenance of form in specific body regions of hydra but also controls the acquisition of normal form in the regenerating hydra and suggest that if growth factors are present, these are at least largely functions of the nervous system. If indeed the nervous system elaborates and contains the growth factors, certain clarifications can be made concerning them. First, that the stimulatory principle is hypostomal in site (Burnett, '61) would depend on the fact that the concentration of nerve cells and neurites in this region exceeds that of the other regions in hydra. Secondly, although Burnett believed that the growth factors were soluble and diffusible in vivo, this is probably not the case. Instead, these compounds are probably restricted in their distribution to elements of the nervous system and could be released at definitive sites. Thirdly, since the stimulating principle appears to be in the present experiments a specific form and growth controlling factor which is delivered to specific sites, it is not necessary to postulate the existence of an inhibitory principle. Thusly, growth and form of hydra would not be a balance between negative and positive factors but only due to positive factors which may vary qualitatively and quantitatively in different regions. Furthermore, the assumed basis for the production of an inhibitory

principle (i.e., high metabolism in the growth and budding region) is questionable, since these areas are no more active for some oxidative enzymes (succinic dehydrogenase, DPNH diaphorase, TPNH diaphorase) and less active for many hydrolases than any other region of hydra (Chapter 3).

It may be more significant that the hydra nervous system appears to contain neurosecretory elements (Chapter 9) and release of these substances at specific sites from neurites controls the fate of that area. On this basis, the present experiments showing the effect of inhibitory neuropharmacological drugs on regeneration could be interpreted as blocking the delivery and discharge of neurosecretory material to effector sites.

Summary

1. The effects of a variety of inhibitory neuropharmacological agents on regenerating hydra were investigated by placing transected hydra in dilute solutions of these agents.
2. Physostigmine, hexamethonium, diisopropylfluorophosphate, atropine, hemicholinium, decamethonium, tubocurarine, reserpine, xylocaine, ergotamine, amphetamine, methyldopate, and chlorpromazine inhibited in varying degrees regeneration and attainment of normal form by transected hydra allowed to regenerate in solutions containing these drugs.
3. Reserpine and chlorpromazine were the most potent

substances tested. Treatment with decamethonium and hemicholinium resulted in a high incidence of bizarre abnormal forms.

4. Transfer of inhibited regenerates to plain media without inhibitors usually resulted in resumption of normal regeneration and eventual attainment of normal form.

5. The nervous system was studied histologically utilizing a methylene blue technique during normal regeneration and in the presence of inhibitory drugs. During normal regeneration, there was a large increase in the number of nerve cells at the regenerating surface 12 hours after transection of the hydra. The large increase in nerve cells at 12 hours did not occur in the presence of the neuropharmacological agents.

6. Distribution and amount of some enzyme activities (acid phosphatase, adenosine-5-phosphatase, and acetylcholinesterase) occurring in normal regeneration were seriously modified and these activities were not reacquired so long as regeneration remained inhibited.

7. These findings indicate that the nervous system of hydra regulates the differentiation and growth of specialized cells in the hydra and the attainment of normal anatomical form of the animal. The inhibitory effects of the neuropharmacological agents on regeneration could be attributed to blocking the delivery and discharge

of neurosecretory material to effector sites.

Fig. 14 Normal hydra (peduncle) stained with reduced methylene blue to demonstrate ganglion cells and neurites. 2075X

Fig. 15 Higher magnification of methylene blue stained ganglion cells. Note stained granules within cytoplasm. 2400X

Fig. 16 Proximal portion stained with methylene blue immediately after transection. Note relative concentration of stained neurons. 1080X

Fig. 17 Regenerating surface of distal half stained with methylene blue two hours after transection. Note decrease in number of neurons at this time. 1200X

Fig. 18 Proximal portion stained with methylene blue after 6 hours of regeneration. Note increase in number of neurons compared with the number present at 2 hours. 1200X

Fig. 19 Regenerating surface of proximal half stained with methylene blue 12 hours after transection. Note large increase in number of neurons. Compare with Figs. 15-17. 480X

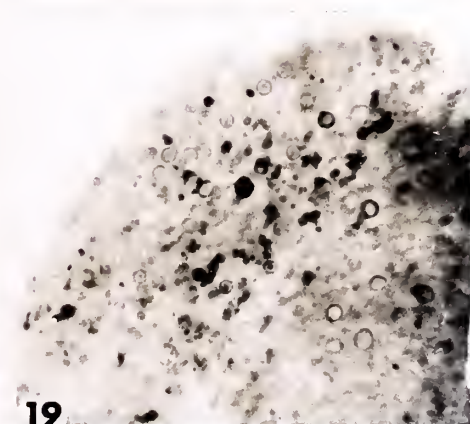
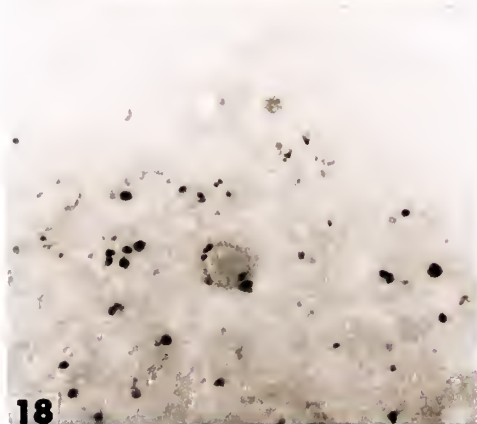
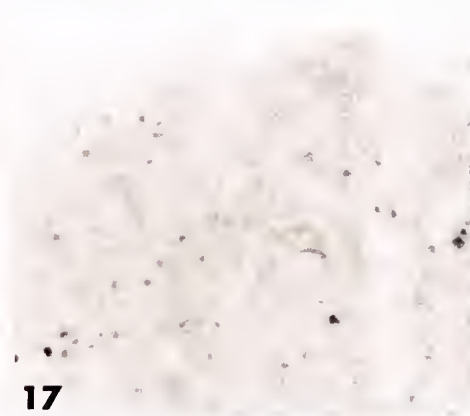
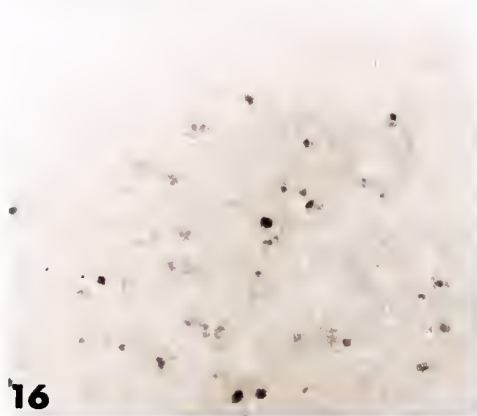
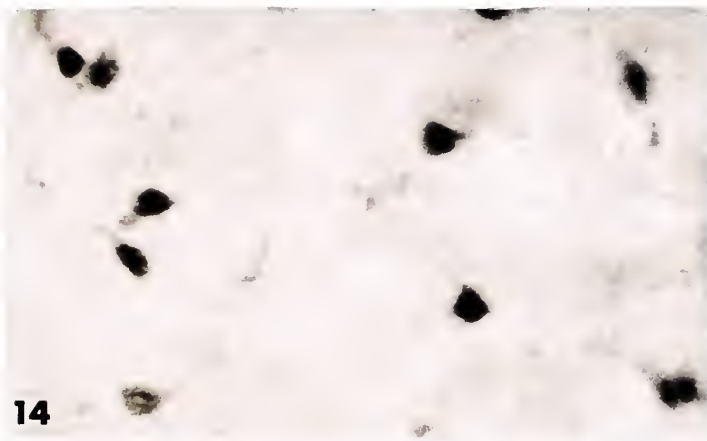


Fig. 20 Proximal portion after 12 hours of regeneration. Note large number of methylene blue stained neurons. 300X

Fig. 21 Distal regenerate stained with methylene blue after 12 hours in DFP ($1.0 \times 10^{-5}M$). Compare with figs. 19 and 20 and note almost complete absence of stained nerve cells. 120X

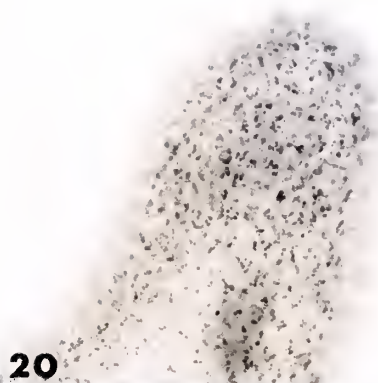
Fig. 22 Distal portion stained with methylene blue after 12 hours of regeneration in reserpine ($1.6 \times 10^{-6}M$). Note scarcity of nerve cells (compare with figs. 19 and 20). 400X

Fig. 23 Proximal portion stained with methylene blue after 12 hours of regeneration in hexamethonium ($3.7 \times 10^{-4}M$). Note paucity of nerve cells compared with normal regenerates (Figs. 19 and 20). 480X

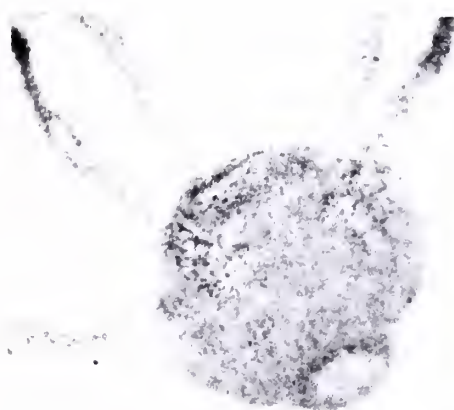
Fig. 24 12 hour proximal regenerate treated with amphetamine ($5.5 \times 10^{-4}M$) (stained with methylene blue). Note that only a few stained cells are present. 400X

Fig. 25 Methylene blue stained distal regenerate after 12 hours in chlorpromazine ($3.5 \times 10^{-5}M$). Note scarcity of nerve cells. 750X

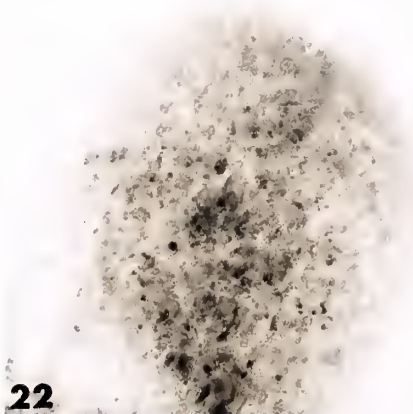
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Fig. 26 Proximal half of transected hydra stained for ALPase activity after 6 days of regeneration in decamethonium ($4.6 \times 10^{-4} M$). The tentacles, peduncle, and base are not intensely reactive as they are in the normal hydra. Only a few loci of cells in the body show intense activity. Note stunted tentacles. 150X

Fig. 27 Distal segment of transected hydra stained for ALPase activity after 6 days of regeneration in physostigmine ($2.4 \times 10^{-4} M$). Note lack of form of the regenerate and the weak intensity of enzyme reaction. 150X

Fig. 28 Proximal segment of transected hydra after 6 days in hexamethonium ($3.7 \times 10^{-4} M$) stained for ALPase. Note the stunted tentacles and beginning formation of two heads. Tentacles show intense activity but peduncle and base remain unreactive. 150X

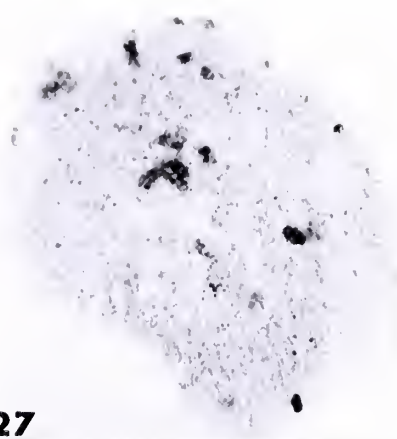
Fig. 29 Distal segment of bisected hydra stained for acid phosphatase activity after 6 days of regeneration in hexamethonium ($3.7 \times 10^{-4} M$). Note unusual form of regenerate. Tips of aborted tentacles show intense activity. 150X

Fig. 30 Proximal portion of a transected hydra stained for acetylcholinesterase after 36 hours of regeneration. Note the abundance of large stained granular neurons. 1200X

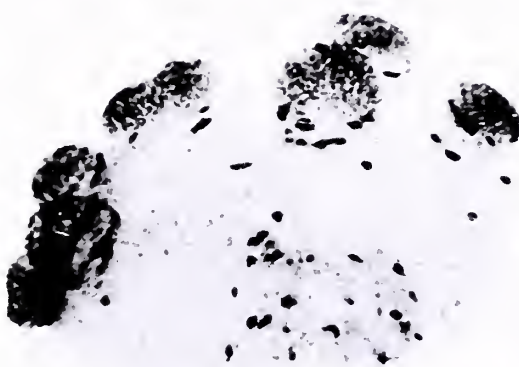
Fig. 31 Proximal portion of a transected hydra stained for acetylcholinesterase after 36 hours of regeneration in DFP ($1.0 \times 10^{-5} M$). Note the almost complete absence of enzymatic reaction indicating a paucity of cells containing the enzyme and/or complete inhibition of the enzyme. 1200X



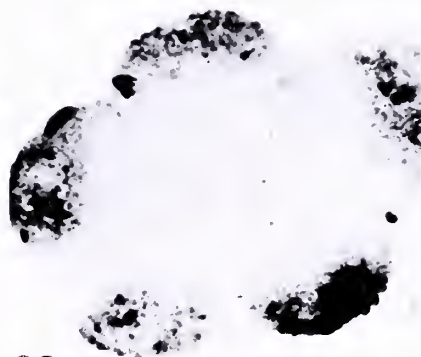
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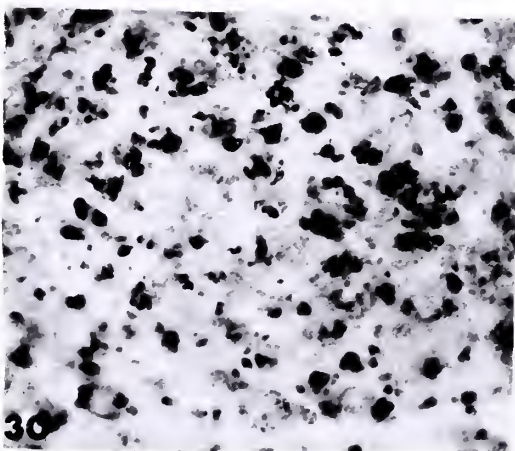
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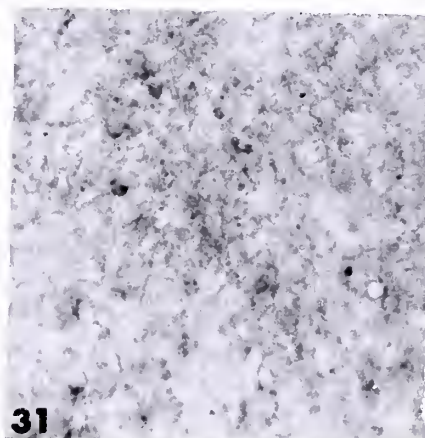
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Chapter 8

Fine Structure of Interstitial Cells and Nematocysts

In the following three chapters, a part of the investigations concerning the fine structural morphology of the hydra are presented. Hydra proved to be remarkably complex and specialized from a fine structural point of view. Sufficient data was obtained from a study of different cell types and different regions of the hydra to preclude its entire presentation here. Therefore, only the fine structural observations related to differentiation, regeneration, and permeability are presented.

This chapter is concerned with interstitial cells and the morphological changes encountered during their differentiation into other cell types, especially nematocysts. Some observations on mature nematocysts are also included.

Materials and Methods

Hydra littoralis were maintained in culture as outlined in Chapter 3. Mature, non-budding hydra which had been fed 24 hours previously were selected for the present studies. These animals, either intact or cut into pieces corresponding to the regions of the hydra (base, peduncle, budding region, stomach and growth region, hypostome and tentacles), were fixed for 1 hour in cold 1% osmium tetroxide buffered with veronal acetate (pH 7.2) and containing 0.4M sucrose (Caulfield, '57) or in cold

4% glutaraldehyde (Sabatini, Bensch, and Barrnett, '63) followed by osmium tetroxide. The fixed tissues were dehydrated with graded concentrations of ethanol and embedded in a 6:4 mixture of butyl and methyl methacrylate, Epon (Luft, '61), or Maraglas (Freeman and Spurlock, '62; Freeman et al., '63). Thick (1 μ) sections of this material were examined with a phase microscope for orientation and thin sections cut on a Forter-Blum microtome were examined with either a Bendix Tronscope or an RCA 3F electron microscope. Some sections were stained with lead hydroxide (Feldman, '62) prior to examination.

Observations

Interstitial cells (Figs. 1 - 5) are totipotent cells capable of differentiating into other cell types. These cells were situated between epitheliomuscular cells above their muscular processes and were most commonly found in the budding region, stomach, and growth region. They occurred predominantly in the epidermis; in fact, few of the undifferentiated forms of these cells were found in the gastrodermis (Fig. 5). Interstitial cells (by definition, undifferentiated) were small and round, about 5 μ in diameter. The cytoplasm of these cells was filled with free ribosomes (Fig. 4). A few small vacuoles and mitochondria with sparse cristae were present. The centrally located nucleus was finely granular and the nucleolus was inconspicuous (Fig. 2). Therefore, it

appears that the small undifferentiated interstitial cells can be characterized by an abundance of ribosomes and a paucity of other cytoplasmic organelles.

As differentiation proceeded, scattered smooth surfaced vesicles increased in number and a Golgi apparatus, consisting of a few smooth surfaced lamellae and small vesicles, appeared in the cytoplasm (Fig. 6). The elements of the Golgi apparatus were sometimes disposed equidistant between the plasma and nuclear membranes, more often closer to the latter, but in no instances was continuity seen with either structure. Mitochondria were not appreciably larger at this stage than before but appeared to be more complex in internal structure (number of cristae) and in some of these cells a few cytoplasmic lipid droplets were apparent. No rough surfaced endoplasmic reticulum was present at this time. Nuclear changes consisted of an increase in size and density of the nucleolus, which was centrally or slightly excentrally located, and an increase in the diffusely arranged chromatin material and frequent indentation of the nucleus.

Following these early relatively undifferentiated stages, the interstitial cells progressively developed through a series of stages into other cell types. There appeared to be an early polarity during the differentiation of interstitial cells into epitheliomuscular cells

(Figs. 7 and 8). One pole of the cell developed several small processes that contained tiny fibrils and extended longitudinally to the body column. The other pole of the cell contained the accumulating cytoplasmic organelles: mitochondria, Golgi apparatus of increasing complexity, and smooth surfaced cisternae. These cells then appeared to increase greatly in size, to enlarge the basal processes and the number of delicate fibrils within them, and to develop the full complement of cytoplasmic constituents including numerous smooth surfaced vacuoles and vesicles. It should be emphasized that the Golgi apparatus and most of the elements of the endoplasmic reticulum were restricted to the apical pole of the cell while the muscular fibrils developed in the base that contained few organelles.

It was quite rare to find interstitial cells differentiating into digestive cells (Fig. 9). However, when observed, the findings were similar to those of epitheliomuscular cells in that the basal pole of the cell (nearest the mesoglea) contained fine fibrillar material and developed processes that extended in a circular direction. The opposite pole of the cell contained mitochondria and an elaborate Golgi apparatus. These cells were different from the epitheliomuscular cells in that they contained in the apical pole a rather well developed smooth surfaced endoplasmic reticulum and cytoplasmic

lipid droplets. At a later stage the smooth endoplasmic reticulum was less prominent in these cells and the cytoplasm was more granular and contained large membrane-bound vacuoles filled with material of several densities.

The development of gland cells from interstitial cells showed interesting morphological features (Fig. 10). Cells intermediate between interstitial cells and gland cells developed an elaborate smooth surfaced endoplasmic reticulum which was circularly oriented and filled the cytoplasm of the cell. These cells also contained lipid droplets. At a later stage, large membrane bound vacuoles filled with homogeneous dense material appeared in the apical cytoplasm of these cells and the cisternae of the endoplasmic reticulum were less regularly arranged and basal in position.

As neurons differentiated from interstitial cells, they acquired processes, a complicated Golgi apparatus, and appeared to elaborate small granules and vesicles (Fig. 11).

During differentiation, interstitial cells were surrounded by the cytoplasm of either differentiated epitheliomuscular or digestive cells (Figs. 5, 7, 9, 10). This finding suggests that a relationship probably exists between the interstitial cell and the enclosing cell that is similar to the relationship of developing spermatozoa to Sertoli cells.

The first stages in the differentiation of interstitial cells into cnidoblasts was accompanied by an increase in the size of the Golgi apparatus and the appearance of a number of smooth surfaced vesicles. Before the appearance of the developing nematocyst, the interstitial cells proliferated into groups of at least two or four cells and even groups of eight have been observed (Figs. 12 - 14). These cells developed in a synchronous manner, probably because the early cells were kept in continuity by intercellular bridges as noted by Slautterback and Fawcett ('59). Two types of interconnections were observed: specialized desmosomal-like thickenings of the limiting plasma membranes bordered by the region of cytoplasmic continuity (Fig. 12) and secondly, by small or large discontinuities of the plasma membranes of two or more adjacent cells (Figs. 12 and 13). The lateral plasma membranes of adjacent cells bordering above and below the interconnections were marked by thickened plates, presumably desmosomal-like in character that were oriented perpendicular to the plasma membrane (Fig. 14).

In a few cases, interstitial cells undergoing mitosis were observed (Figs. 13 and 15). Long chromosomes, often paired, were present within the cytoplasm. The chromosomes were composed of finely granular dense material. Thin transverse lines, usually paired, within the chromosomes may represent genetic loci (Fig. 15).

Following the stage of proliferation, the capsule of the nematocyst was seen first as a small round mass of homogeneous material of light density within a large dilated membranous saccule and completely surrounded by smooth surfaced lamellae and vesicles of the Golgi apparatus. The capsule apparently grew by fusion of large and small Golgi vesicles to the membrane surrounding the nematocyst (Fig. 16). In this manner the contents of the Golgi vesicles could be deposited within this developing structure. Many developing nematocysts were surrounded by small, evenly spaced vesicles approximately 200\AA in diameter. These structures were connected to the outer membrane of the capsule by a narrow tubule (Figs. 17 - 19).

During the early stages of formation of the nematocyst, cytoplasmic vesicles first increased in number and then coalesced to form circularly-oriented lamellae of endoplasmic reticulum which was initially free for the most part of associated ribosomes. The endoplasmic reticulum continued to develop until it practically filled the entire cytoplasm of the cnidoblasts. It later acquired ribonucleoprotein particles on the outer aspect of the membranes of the lamellae (Figs. 17 - 19). Mitochondria were interspered sparsely among profiles of the reticulum and the elements of the Golgi apparatus were prominent and closely positioned in relation to at least

one surface of the developing nematocyst (Fig. 20). The nucleolus was also large and dense at this time (Fig. 22).

During these changes in cytoplasmic constituents of the cnidoblast, several changes were noted in the nematocysts which continued to increase in size throughout the entire process. Early in development dense material appeared in the apex and center of the nematocyst and this material was surrounded by the homogeneous material of lighter density that was deposited earlier (Figs. 21 and 22). These materials increased in amount as the nematocysts grew until the apex and central core consisted of a relatively dense material and the less dense homogeneous material formed a capsule around it. About the periphery of the latter material, a smooth surfaced membrane formed the limits of the nematocyst. In addition, within the denser material short filaments and very dense granules appeared during the stage in which the endoplasmic reticulum attained its full development (Figs. 20 - 22). The filaments and very dense granules increased in amount and were initially arranged roughly clumped in a circumferential manner or homogeneously dispersed. Since the density of these latter elements and those of the fully formed stylets and spines were quite similar, it is suggested that they are indeed the unaggregated precursor material of the harpoon-like structure that occupies the center of the fully formed nemato-

cyst.

The remaining stages of differentiation involved the development of intracapsular structures and the regression of cytoplasmic organelles. In the cytoplasm, the lamellar organization of the endoplasmic reticulum broke up into isolated vesicles and ribosomal granules. The Golgi apparatus also regressed and became an inconspicuous body at one side of the nucleus, which was flattened and pushed to the periphery of the cnidoblast (Fig. 23).

It was difficult to obtain examples depicting the actual course of development of the spines, stylets, thread, and operculum of the nematocyst. These events must have occurred very rapidly because of the relative scarcity of intermediate morphological stages. Segments of stylets and spines appeared within the granular matrix of the nematocyst and were separated from the matrix by a space of light density that was continuous with the capsular material. Apparently the segments of dense material were then organized within the nematocyst to produce the fully formed harpoon-like structure of stylets and spines. Similarly, the thread appeared to be formed by the appearance of material of light density surrounding a denser core of intracapsular granular material.

The mature stenotele was composed of a capsule of low density material surrounding the intracapsular struc-

tures (Figs. 23 and 24). The operculum or lid was situated at the apical end of the nematocyst and was of equal density as the capsule and continuous with it at one point. The contents of the capsule were made up of a finely granular matrix, stylets, spines, thread, and invaginated capsular wall. The stylets were large rod-like structures of very high density and converged at the apical end of the nematocyst (Fig. 24). The stylets surrounded the smaller and more numerous spines which also pointed toward the apex of the nematocyst (Fig. 25). The thread was wound about in the basal portion of the nematocyst (Fig. 24) and in section, it bore the shape of a three-bladed propeller (Fig. 26). The thread, stylets, and spines were surrounded with a layer of low density material that was continuous with the capsular wall at the apex. The portion of this invaginated capsular wall surrounding the stylets and spines was 800 \AA in diameter and divided into regular 200 \AA units (Fig. 27). The portion of the invaginated capsular wall that surrounded the thread and actually formed the outer wall of the thread was only 200 \AA in diameter (Fig. 26). Rarely, small fibrils extending between the capsule and the base of the cell were observed in the basal portion of the cnidoblast.

The other types of nematocysts were basically the same in structure as the stenoteles with minor variations.

Desmonemes ($4 \times 5 \mu$) were smaller than stenoteles ($9 \times 14 \mu$), lacked stylets and spines (Fig. 28), and possessed a large and tubular thread. Isorhizas were more elaborate in shape ($2 \times 5 \mu$) than the other types (Fig. 29). Holo-trichous isorhizas differed from atrichous isorhizas in that they bore spines on the inner aspect of the coiled thread while both types lacked the elaborate harpoon-like structure observed in the stenoteles.

The cnidocil, which has been described by Bouillon and his co-workers ('58) and Chapman and Tilney ('59), was surrounded by a limiting membrane continuous with the plasma membrane of the cnidoblast. Twenty or 21 dense rods appeared to arise from the capsule of the nematocyst and extend upward (Figs. 30 and 31). These rods, known as the supporting rods of the cnidoblast, were composed of two concentric cores of dense material. Distally, these structures gave rise to the larger irregularly-shaped supporting rods of the cnidocil (Figs. 30 - 32). The outer membranes of both structures were continuous at one point. The supporting rods of the cnidocil separated from the rods of the cnidoblast and extended to the terminal portion of the cnidocil (Fig. 33). The center of the cnidocil was occupied by a dense core surrounded by nine small rods suggesting that this structure is in reality a modified cilium or flagellum.

Cnidoblasts were most numerous in the tentacles,

where several were embedded in each epitheliomuscular cell. Mature nematocysts were located primarily in the tentacles, although a few were present in other regions, especially the peduncle. The stomach region contained large numbers of developing nematocysts in all stages of differentiation.

Discussion

The interstitial cells, the least differentiated cell type in the hydra, contained few cytoplasmic organelles and an abundance of free ribosomes. These cells, because of their ribosomal content, may be capable of rapid protein synthesis, presumably during their differentiation into definitive cell types. Some features were common to the differentiation of interstitial cells into other types. The earliest stages were characterized by elaboration and development of the Golgi apparatus and endoplasmic reticulum. In the case of cnidoblasts and nerve cells, the Golgi apparatus continued to develop but the endoplasmic reticulum reached its highest development during differentiation of cnidoblasts and gland cells. The development of epitheliomuscular and digestive cells was characterized by increase in size and the elaboration of cytoplasmic vacuoles and muscular processes.

The differentiation of interstitial cells into cnidoblasts provides an excellent model for the study of cell differentiation and the elaboration of a complex

organelle. These results reemphasize the findings of Slautterback and Fawcett ('59) and Slautterback ('61). Two types of intercellular bridges were noted early in the development of the interstitial cell into a cnidoblast. The continuity of cell probably serves to synchronize the development of groups of cells, especially in view of the observations that within one group, all nematocysts were of the same type and at the same stage of development. Nematocyst formation appears to begin within the Golgi apparatus in close proximity to the nucleus. Enlargement of the primordial nematocyst was apparently accomplished by deposition of material from numerous Golgi vesicles surrounding the periphery of the nematocyst. The endoplasmic reticulum developed simultaneously with the beginning formation of the nematocyst, appearing first as vesicles and developing into a complex lamellar structure with numerous ribosomes attached to the other surfaces of the membranes. This stage is probably one of maximum protein synthesis and coincides with the greatest increase in size of the nematocyst. Presumably, protein material elaborated by the complex endoplasmic reticulum is channelled through the Golgi apparatus before being deposited onto the nematocyst. The endoplasmic reticulum and Golgi apparatus regressed when the nematocyst reached its definitive size, although the inner structures of the nematocyst were not fully

differentiated. The nucleolus was also fully developed at this stage and may play some role in protein synthesis. The remainder of differentiation was concerned with the intracapsular development of spines, stylets, and thread.

Slautterback and Fawcett ('59) and Slautterback ('61) state that the thread is formed outside of the capsule and is later withdrawn into the capsule. Weill ('34), Hyman ('40), and Chapman and Tilney ('59) on the other hand, postulate on the basis of light microscope observations that the thread is formed within the capsular matrix. Weill ('34) furthermore stated that the immature nematocyst is capable of evaginating. The present work supports with fine structural observations the latter interpretation, since no stages were observed in which the thread was being withdrawn into the capsule. Furthermore, it appeared that the thread was elaborated within the capsule along with the stylets and spines.

These observations on the structure, distribution, and differentiation of interstitial cells provides additional information concerning the role of these cells in the growth processes of hydra. The greatest concentration of interstitial cells was in the distal end of the stomach, the area called the growth region. The interstitial cells are capable of differentiating into epitheliomuscular cells, digestive cells, gland cells, and neurons as observed by previous workers (Brauer, 1891;

Schultz, '18; Gelei, '24, '25; Strelin, '28; Kanajew, '30; McConnell, '32). These comprise all the cell types of hydra with the exception of epithelial cells of the base which appear to develop exclusively from epitheliomuscular cells of the peduncle. In the present work intermediate stages between interstitial cells and other cell types were observed not only in the growth region but in the hypostome, the stomach, and the budding region. This observation supports Burnett's view ('62) that cells of specialized types in areas other than the extremities are replaced by differentiation of interstitial cells located anywhere along the body. The loss of individual cells at the extremities, on the other hand, is restored primarily by proximal and distal migration of dividing epitheliomuscular and digestive cells from the growth region. Gland cells, cnidoblasts, and neurons appear to develop exclusively from interstitial cells. Brien and Reniers-Deceon ('55) have shown that irradiated hydra which have lost their interstitial cells gradually lose their gland cells and cnidoblasts and eventually die. Gland cells are replaced in the hypostome where they are most concentrated and appear to migrate but are usually expended before reaching the tentacles or base. Interstitial cells differentiated into neurones primarily in the hypostome and a few were found present in the stomach, the budding region, and the peduncle.

It appears that neuron replacement in the hypostome is accomplished by differentiation of interstitial cells while those lost at the base must be replaced by proximal migration of either mature neurons or intermediate cells. Although interstitial cells differentiated into epitheliomuscular and digestive cells, the predominant mechanism of replacement is mitosis of these cells in the growth region (Burnett, '62).

Differentiation of interstitial cells into cnidoblasts usually occurred in the growth region. Cnidoblasts then must migrate from the growth region through the hypostome and into the tentacles, since differentiation of interstitial cells in the tentacles does not account for the large number of mature nematocysts present in that region. This migration may occur in three ways. First, individual cnidoblasts may move between epithelial cells into the tentacles. Lenhoff ('59) found that nematocysts labeled with ^{14}C moved from the stomach to the tentacles in two days. To reach the tentacles in such a short time, cnidoblasts would have to actively migrate to the tentacles. Secondly, cnidoblasts could be carried to the tentacles within epitheliomuscular cells which move via the normal growth processes (Brien and Reniers-Decoen, '50; Semal-Van Gansen, '51; Burnett and Lentz, '60). Because most cnidoblasts were embedded within epithelial cells, this may be the

major means of migration under normal conditions. Finally, Hadzi ('07), Kepner et al., ('37), Jones ('41), and Burnett ('60) suggested that cnidoblasts pass through the gastrodermis into the digestive cavity and are then carried to the tentacles where they migrate through the gastrodermis again into the epidermal layer. However, very few nematocysts were seen within the gastrodermis of the stomach and none in the tentacles. Those present within gastrodermal cells were being digested.

With a better knowledge of the normal differentiation processes, the role of interstitial cells in regeneration can be appraised. First, it appears that interstitial cells are necessary for the ultimate survival of regenerating hydra. Basal disks chopped into a tissue mince are sometimes capable of reconstituting general form although they lack interstitial cells and also do not survive (Burnett, '61). Lacking interstitial cells, the hydra is able to survive as long as it has a supply of the specialized cell types. However, once the cells present are spent, the hydra succumbs being unable to replace them. It appears, therefore, that specialized cell types are incapable of dedifferentiating morphologically into interstitial cell although it was shown (Chapter 3) that enzymatic dedifferentiation occurs during regeneration.

The nematocyst is a remarkably complex structure

specialized for immobilization and killing of the hydra's prey. A thick homogeneous capsule surrounds the spines, stylets, and threads. The spines and stylets are enclosed by the invaginated capsular wall and the thread is continuous with the latter. During eversion, these structures must be completely turned inside out since after discharge the spines and stylets protrude exteriorly from the now evaginated capsular wall and the thread points outward.

The cnidoblast contains a small Golgi apparatus, a few mitochondria, and a nucleus compressed to one side of the cell. The once highly developed endoplasmic reticulum remains as only a few scattered vesicles. The sole function of this cell has been the production of the nematocyst and once the nematocyst is discharged, the cell presumably dies.

The fine structure of nematocysts may provide some information concerning nematocyst discharge (see Chapter 5 for further discussion). At present it is assumed by the majority of workers that nematocysts are independent effectors which require a combination of chemical and mechanical stimulation for eversion. However, the nervous system may play a significant role in nematocyst discharge acting as a subliminal mechanism in addition to the effects of mechanical and chemical stimuli (Chapter 5). Following appropriate stimulation, the

nematocyst is discharged either by contraction of the capsule causing eversion of the tube (Jones, '47) or by swelling the capsule contents by intake of water (Yanagita, '43; Yanagita and Wada, '54; Ficken, '53, '57; Robson, '53).

The present studies showed the cnidocil to be continuous with the capsule of the nematocyst. This relationship makes it possible for stimuli, either mechanical or chemical, to reach the nematocyst from the exterior cnidocil. Furthermore, a number of enzymes are present within the cnidocil and capsule of the nematocyst (Chapter 3) and presumably a chain of enzymatic events occur between stimulation of the cnidocil and discharge of the nematocyst.

Summary

1. The fine structure of interstitial cells, the morphological changes encountered during their differentiation into other cell types, especially nematocysts, and observations on mature nematocysts are presented.
2. Small undifferentiated interstitial cells were characterized by an abundance of ribosomes and a paucity of other cytoplasmic organelles.
3. Early stages of differentiation of interstitial cells into other cell types were characterized by an increased number of cytoplasmic vesicles, the appearance of a Golgi apparatus, and the increased size and density of the nucleo-

lus.

4. The Golgi apparatus continued to develop in the case of cnidoblasts and nerve cells and the endoplasmic reticulum reached its highest development during differentiation of cnidoblasts and gland cells. Epitheliomuscular and digestive cell development was characterized chiefly by increase in size and elaboration of cytoplasmic vacuoles and muscular processes.

5. Nematocyst development was marked by synchronous development of cells connected by intercellular bridges, elaboration of a complex lamellar, rough-surfaced endoplasmic reticulum, and enlargement of the primordial nematocyst by deposition of material from numerous Golgi vesicles surrounding the periphery of the nematocyst. This stage is probably one of maximum protein synthesis and coincides with the greatest increase in size of the nematocyst. Presumably, protein material elaborated by the complex endoplasmic reticulum is channelled through the Golgi apparatus before being deposited onto the nematocyst.

6. The remainder of differentiation was concerned with the intracapsular development of spines, stylets, and thread and the regression of the endoplasmic reticulum and Golgi apparatus.

Abbreviations for Electron Micrographs

C	capsule	MV	microvillus
CC	central core	N	nucleus
EFL	external fibrillar layer	NC	nerve cell
ER	endoplasmic reticulum	Ne	nematocyst
EPL	external protective layer	Nl	nucleolus
F	lipid droplet	NP	nuclear pore
FV	food vacuole	NS	neurosecretory granule
G	Golgi apparatus	O	operculum
GC	ganglion cell	P	nerve cell process (neurite)
Gl	gland cell	PV	pinocytotic vesicle
Gly	glycogen	S	secretory droplet
I	interstitial cell	SC	sensory cell
ICS	intracellular space	SCb	supporting rod of cnidoblast
ICW	invaginated capsular wall	SCn	supporting rod of cnidocil
M	mitochondrion	Sp	spines
Me	mesoglea	St	stylets
Mf	myofibrils	T	thread (filament)
MG	mucous gland cell		
MP	muscular process		
MT	microtubule		
Mu	mucous granule		

Fig. 1 Four interstitial cells. The two cells on the bottom are undifferentiated while those on the top are slightly differentiated possessing an increased number of cytoplasmic vesicles. Note that the differentiating cell on the top also possesses a dense nucleolus and a large cytoplasmic lipid droplet. All the cells are embedded within epitheliomuscular cells and situated above their muscular processes. 16,000X

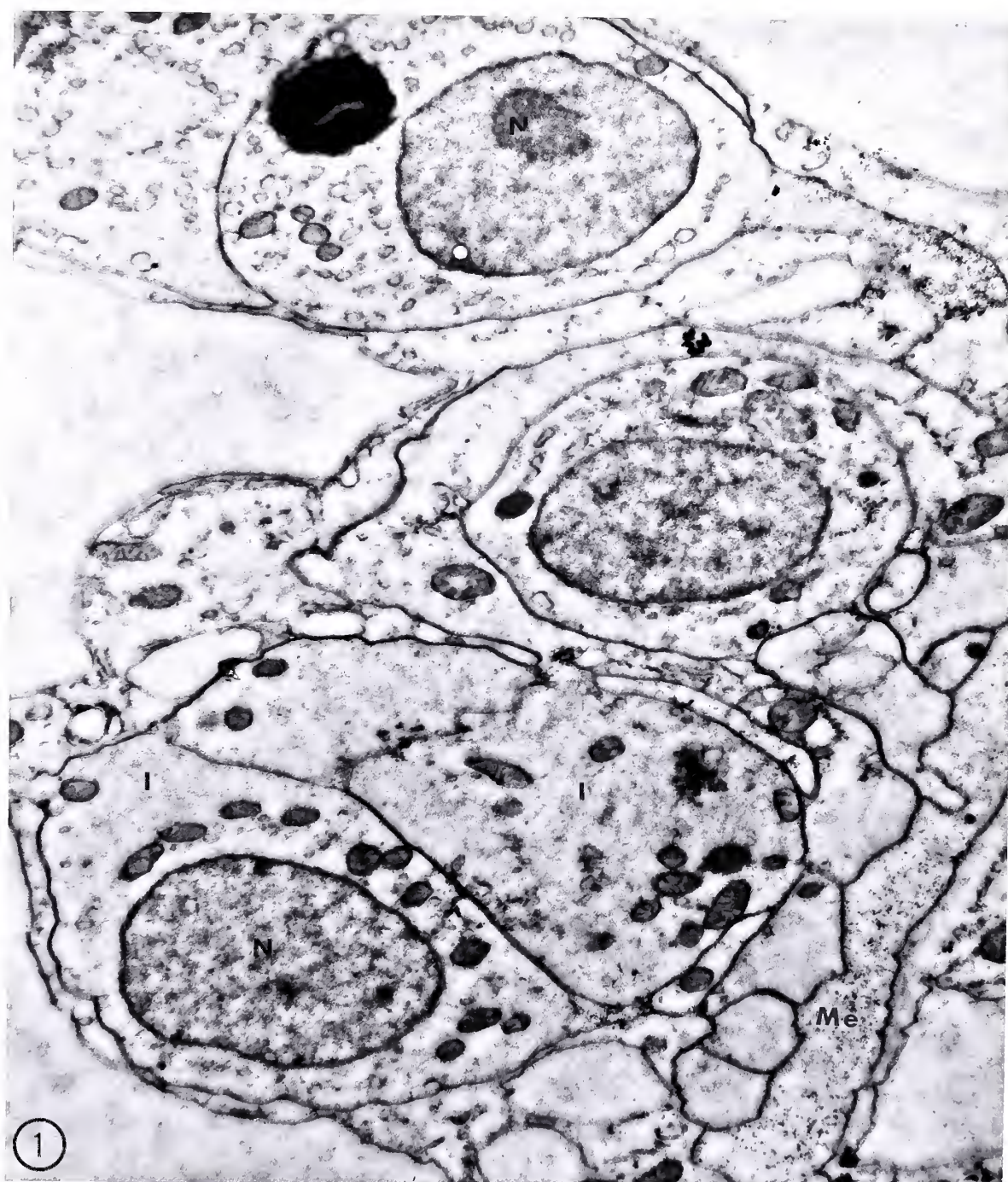


Fig. 2 Interstitial cell. Note the large nucleus and incospicuous nucleolus. The cytoplasm contains a few mitochondria and numerous ribosomes. The cell is slightly elongated suggesting that it is migrating between other cell types. 33,000X

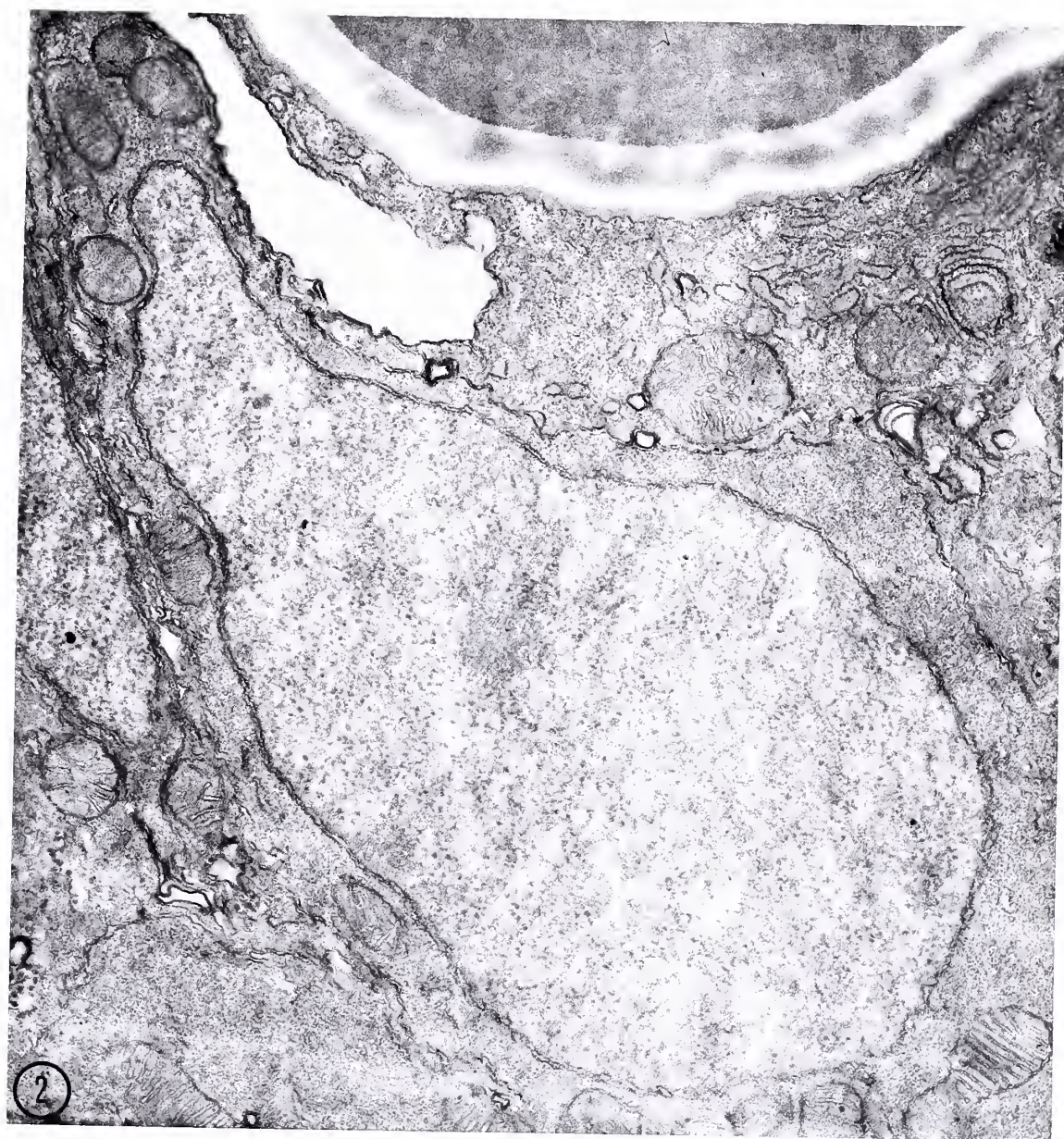


Fig. 3 Two interstitial cells. Mitochondria and ribosomes are present in the cytoplasm. Cytoplasmic vesicles are also present indicating these cells are in the earliest stages of differentiation. Note the large nuclei and inconspicuous nucleoli. 29,000X

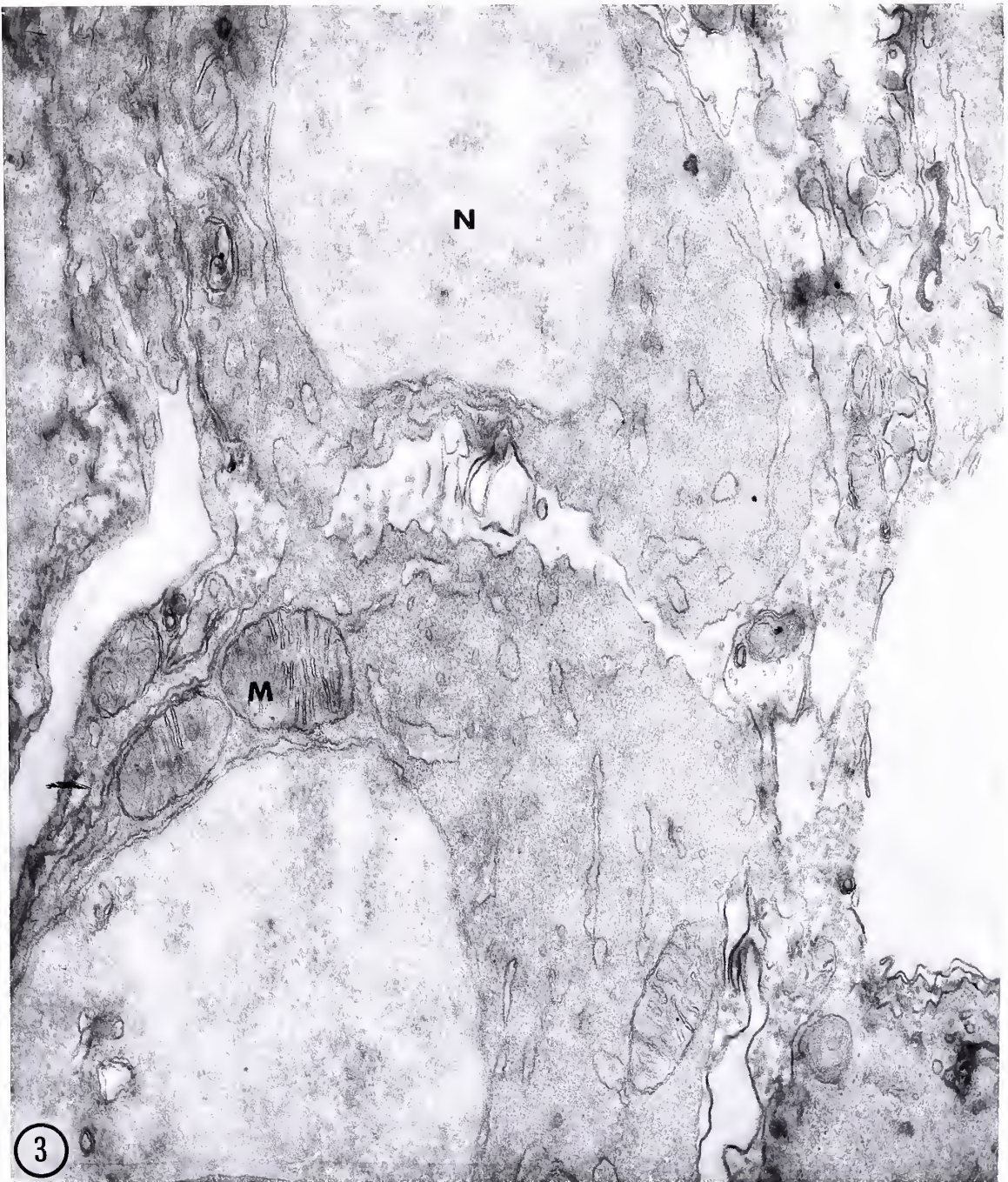


Fig. 4 High magnification of the cytoplasm of an interstitial cell. Note the abundance of ribosomes. Mitochondria are present within the cytoplasm. A nucleus and nucleolus are also present. The nucleus is surrounded by a double membrane. 45,000X

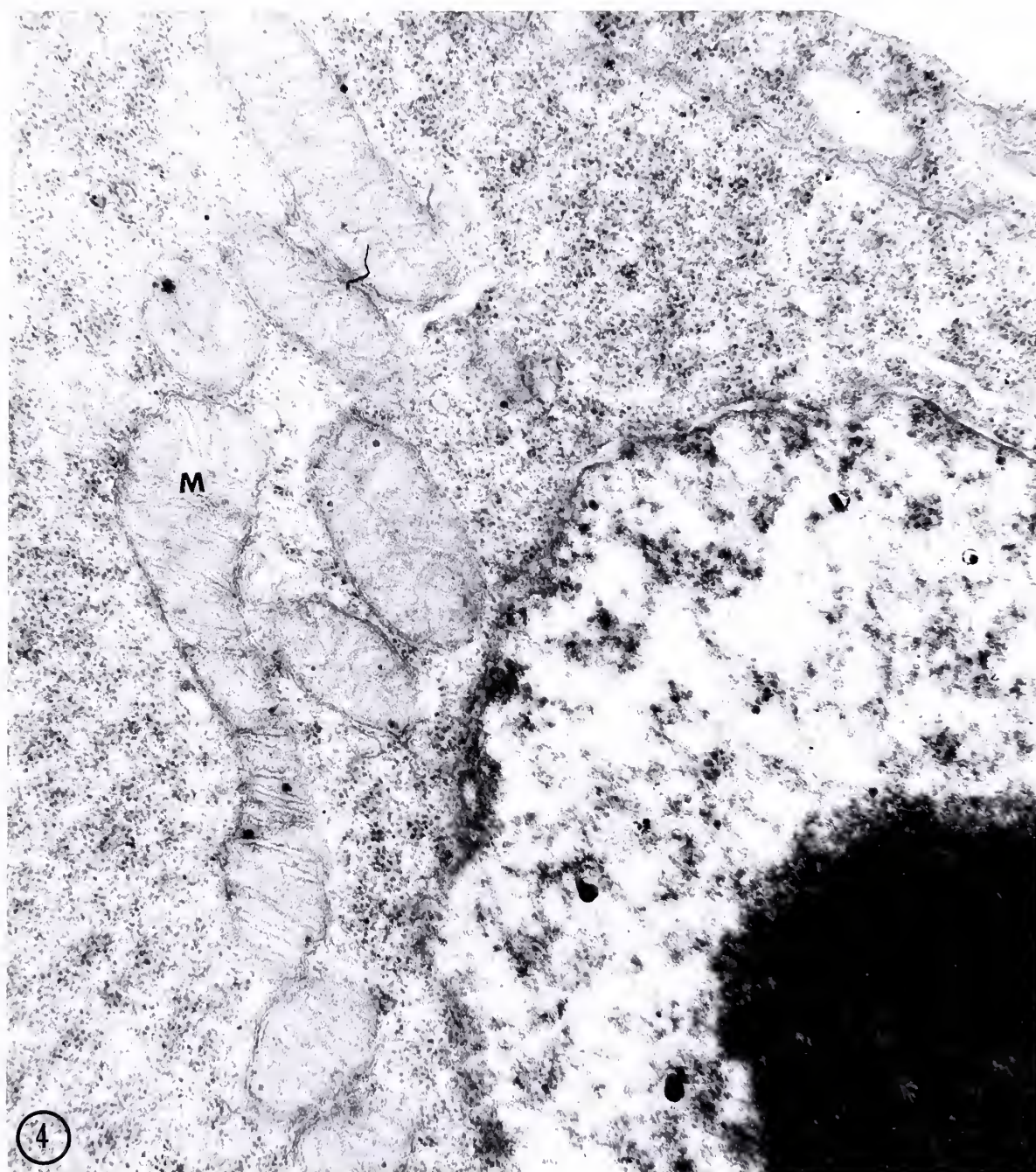


Fig. 5 Interstitial cell within the gastrodermis.
note the fat vacuoles within the cytoplasm. A thin
layer of cytoplasm belonging to a digestive cell
surrounds the interstitial cell. 12,000X

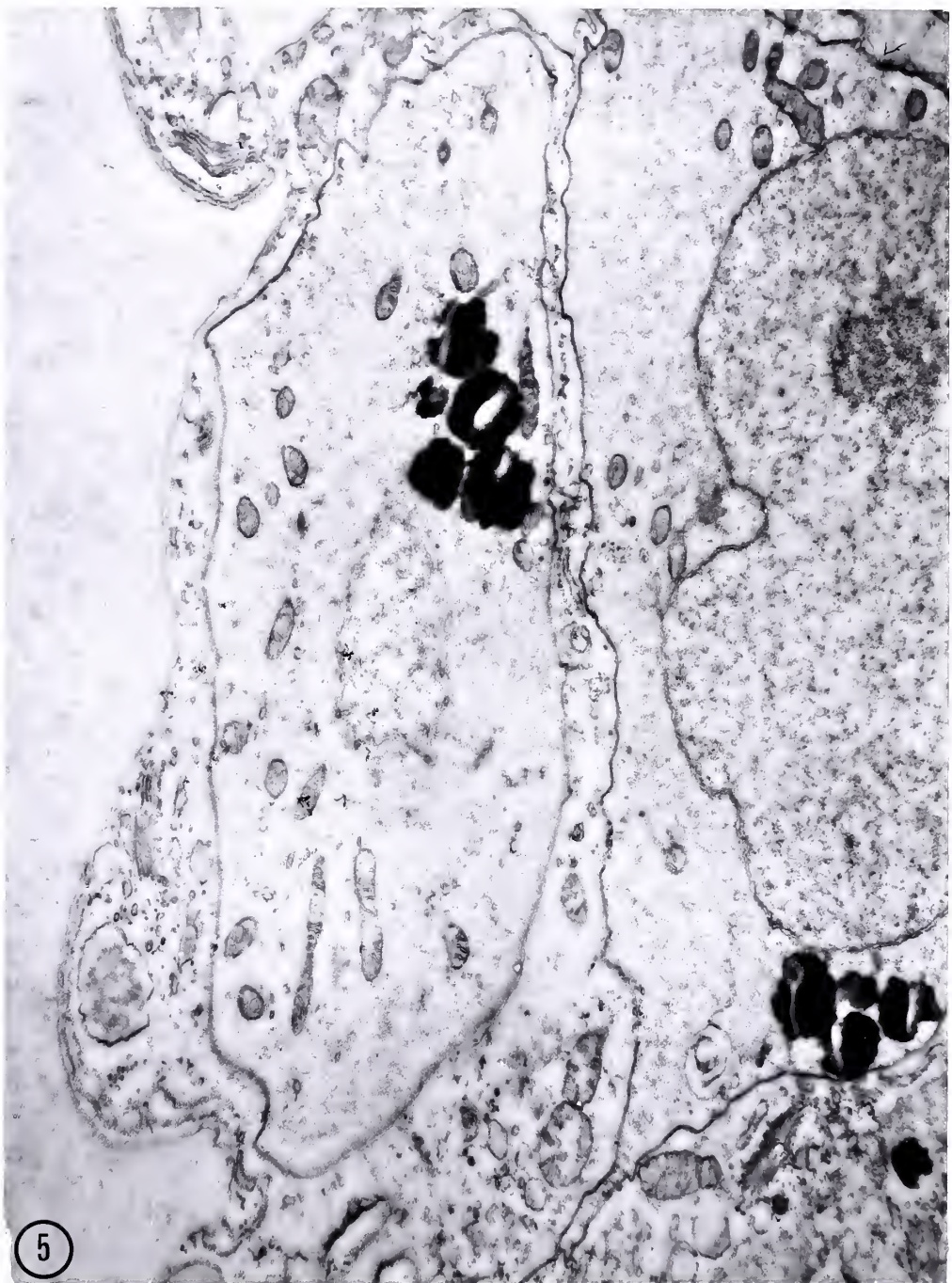


Fig. 6 Interstitial cell in an early stage of differentiation. A Golgi apparatus, mitochondria, numerous ribosomes, and vesicles are present in the cytoplasm. The cell is surrounded by a thin layer of cytoplasm from an epitheliomuscular cell. 29,000X

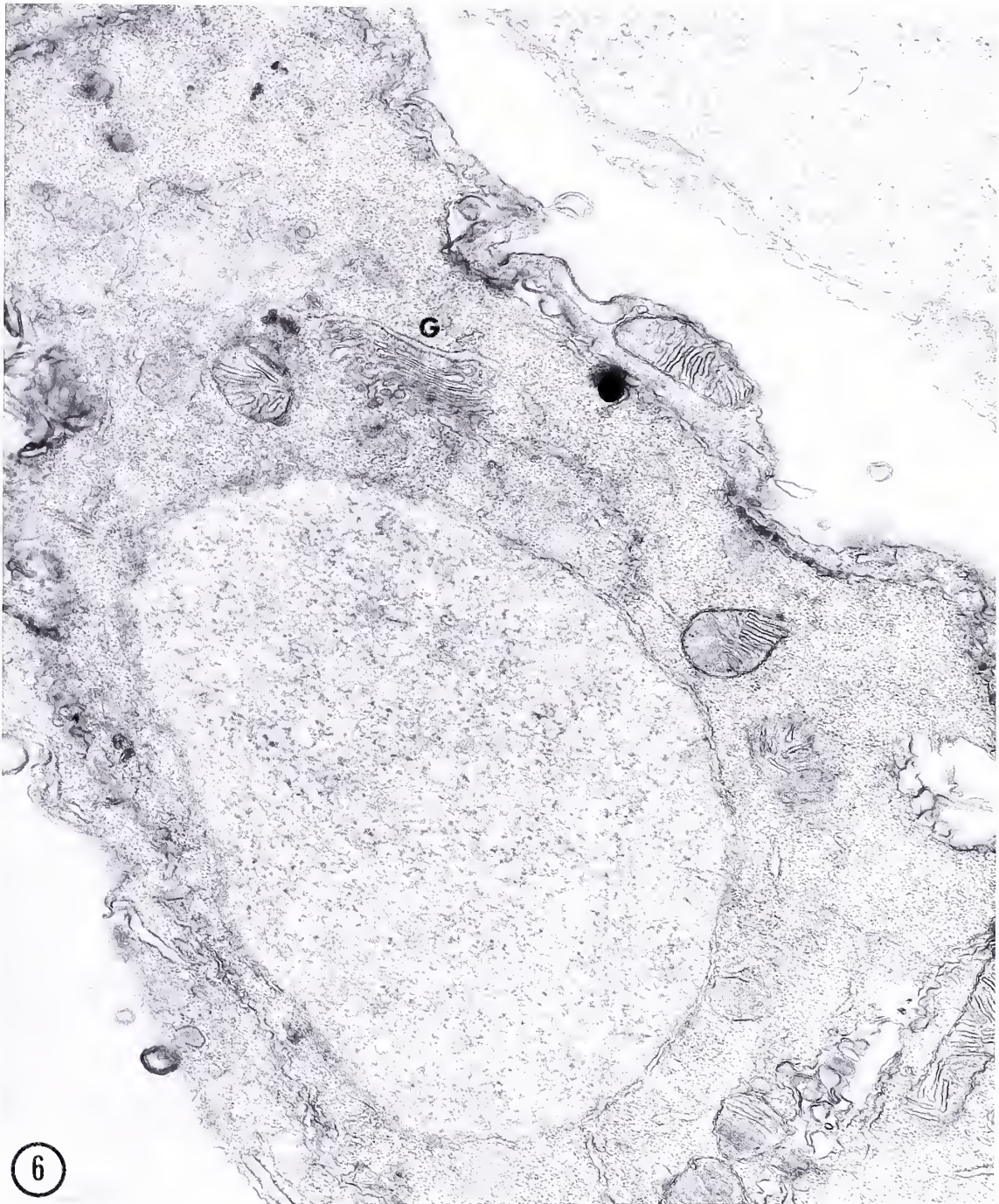


Fig. 7 Intermediate stage in the development of an interstitial cell into an epitheliomuscular cell. Note the small size of the cell in relation to the mature epitheliomuscular cell surrounding it. The developing cell possesses a small process containing fibrils. The cytoplasm contains an increased number of vesicles in comparison to an undifferentiated interstitial cell. 19,000X

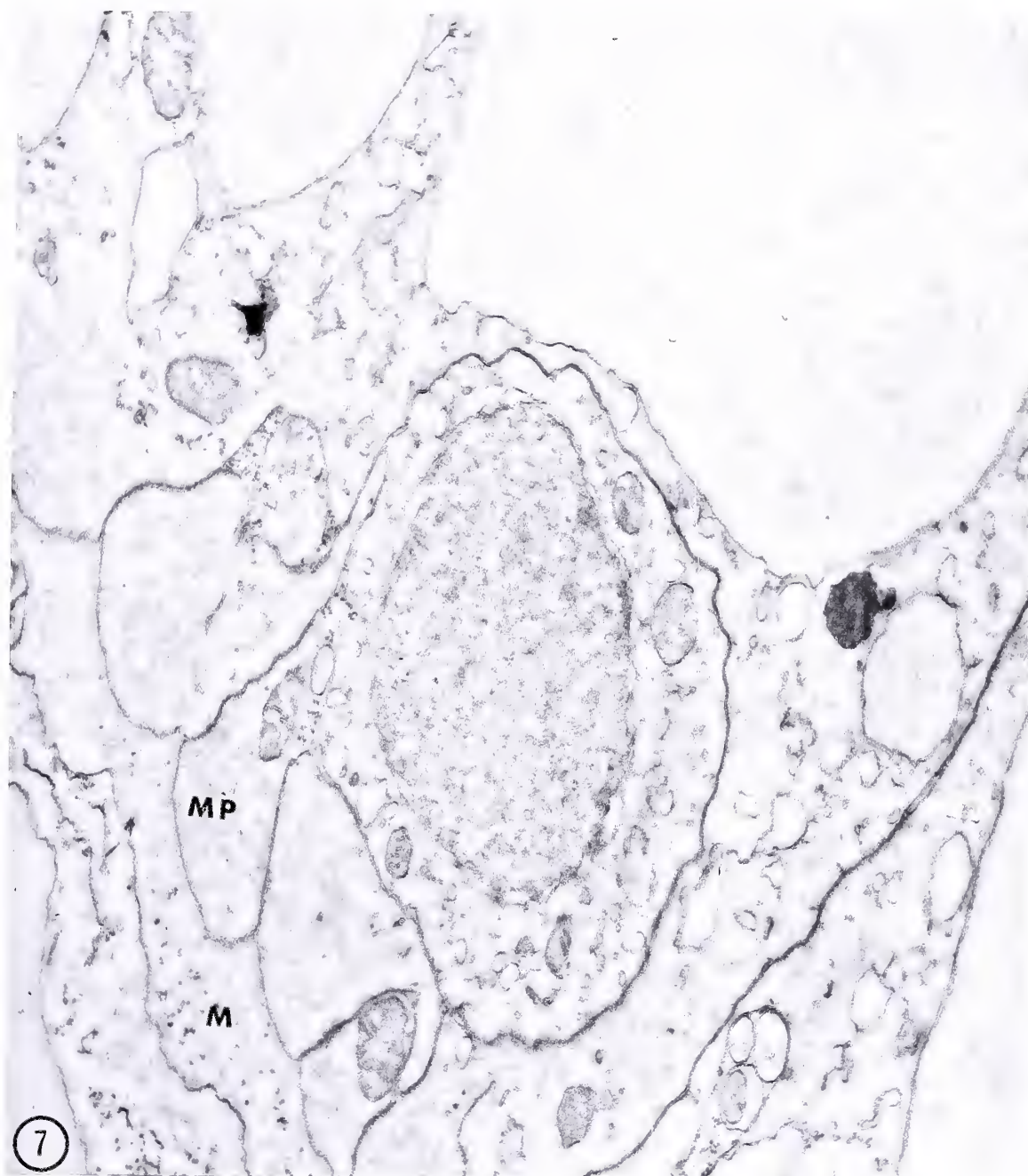


Fig. 8 Two interstitial cells lying above the muscular processes of epitheliomuscular cells. The cell on the right is undifferentiated containing only ribosomes and mitochondria within the cytoplasm. The cell on the left contains cytoplasmic fibrils indicating it is differentiating into an epitheliomuscular cell.

21,000X

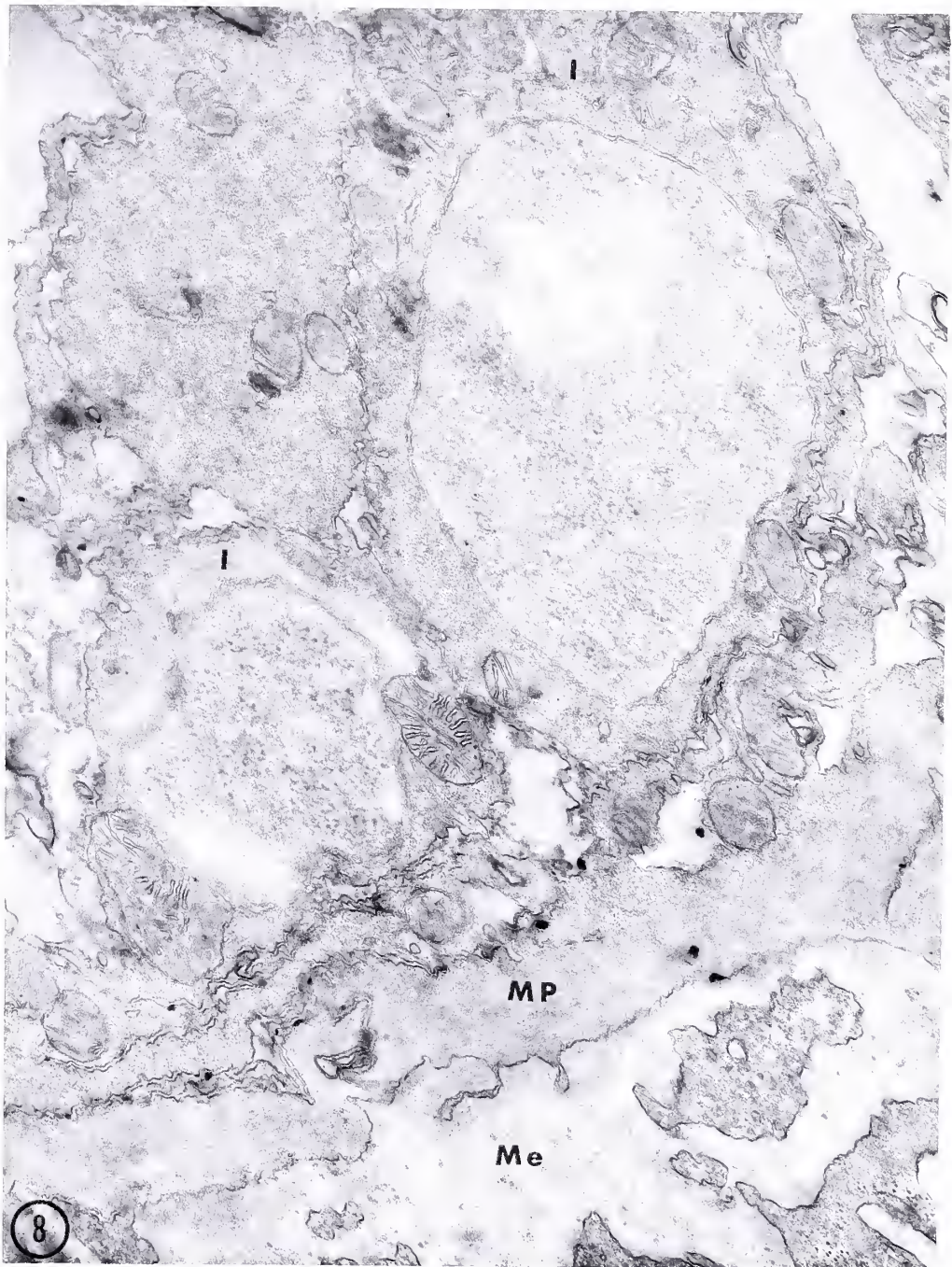


Fig. 9 Intermediate stage between interstitial cell and digestive cell. The cytoplasm is beginning to differentiate containing a Golgi apparatus, mitochondria, and smooth-surfaced vesicles. Fibrils are present in the cytoplasm at one pole of the cell. Note that the entire cell is surrounded by a thin process of a digestive cell. The cell lies above the muscular processes of digestive cells. 12,000X

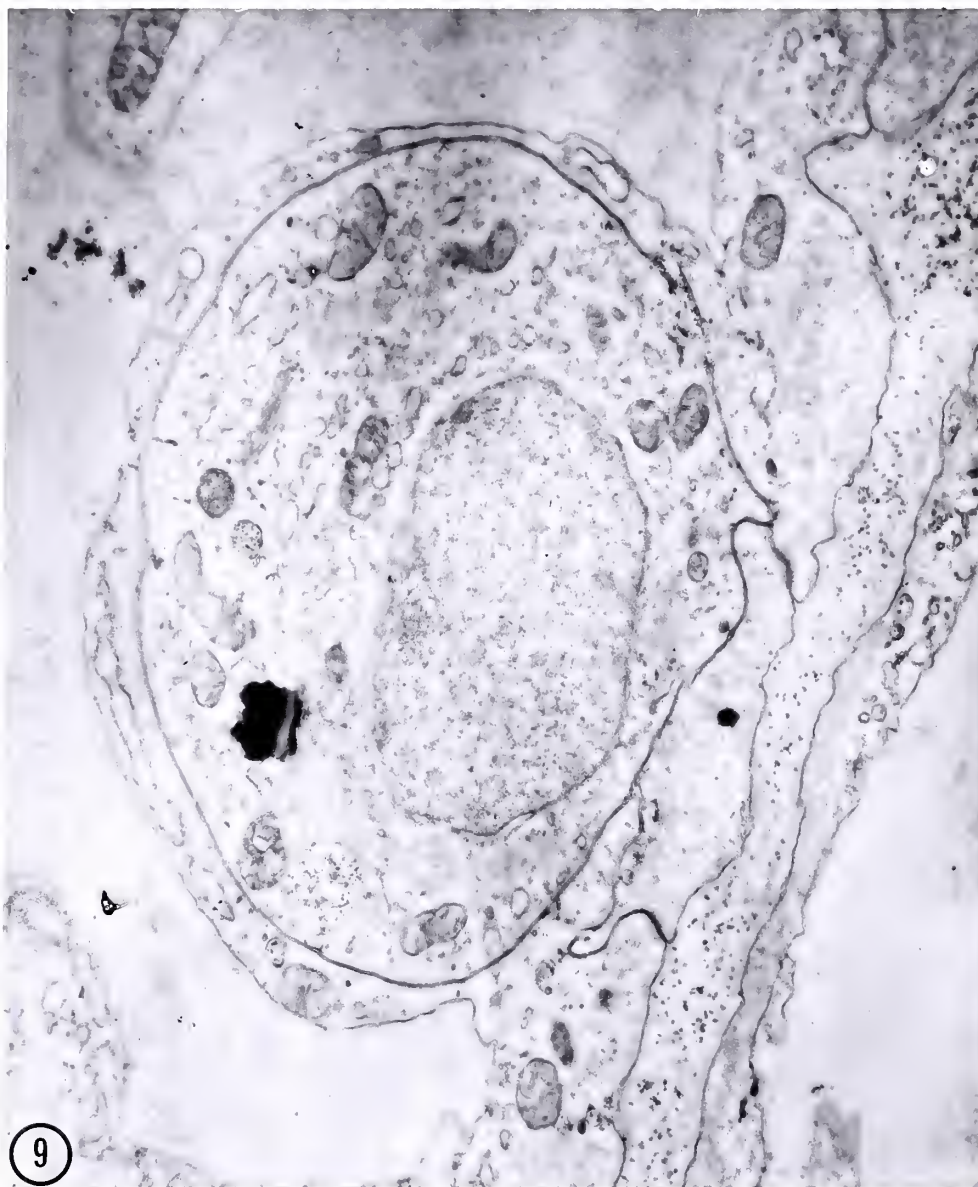


Fig. 10 Intermediate stage in the development of an interstitial cell into a gland cell. Note the elaborate tubular endoplasmic reticulum situated circularly around the nucleus. Fat droplets are present in the cytoplasm. The cell is enclosed by a digestive cell. 12,000X

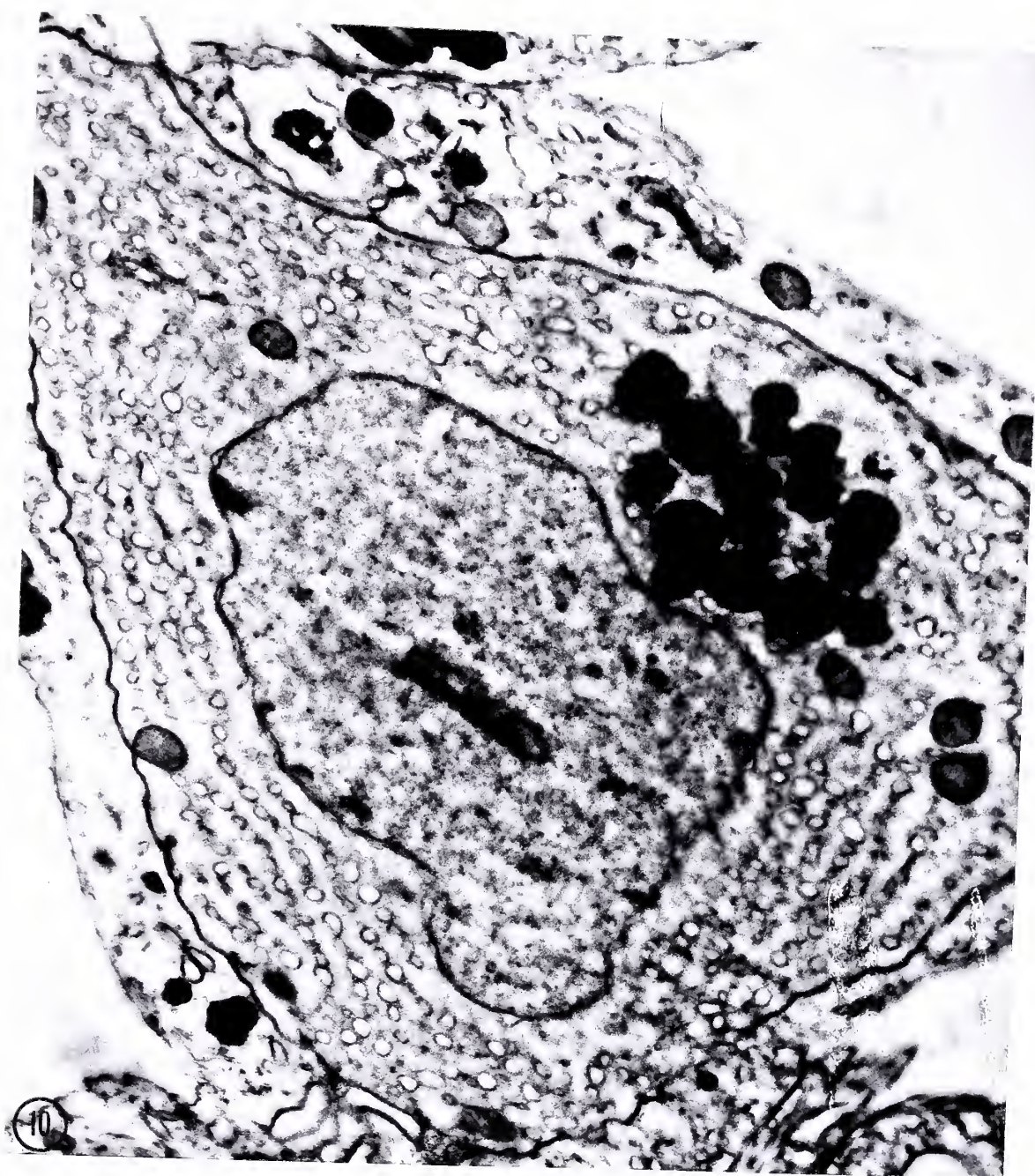


Fig. 11 Intermediate stage in the differentiation of an interstitial cell into a neuron. The cell is identified by the elaborate Golgi apparatus characteristic of neurons. Mitochondria are large and numerous. The cytoplasm is filled with ribosomes.

44,000X

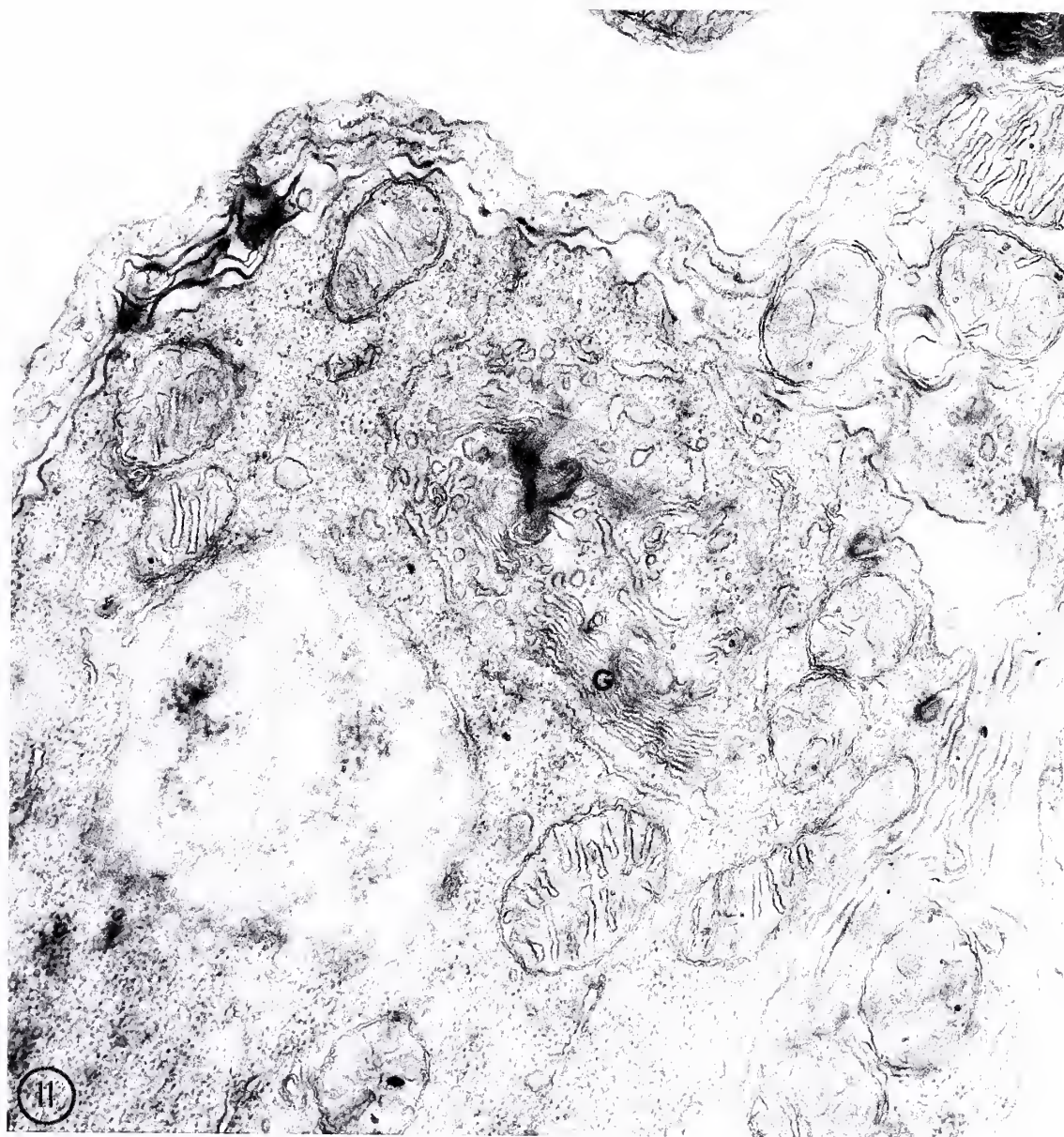


Fig. 12 Differentiating interstitial cells. Note that the cells which are continuous because of discontinuities in cell membranes (bars) are in the same stage of differentiation. The endoplasmic reticulum is represented by numerous cytoplasmic vacuoles. The nucleoli of the differentiating cells are dense and large whereas no nucleolus is apparent in the adjacent undifferentiated interstitial cell. 7,000X



Fig. 13 Four interstitial cells undergoing mitosis. The chromosomes are apparent as dense, elongated, and occasionally paired structures in the cytoplasm. Note that no nuclear membrane is present. Mitochondria are present within the cytoplasm. Discontinuities are present in the cell membranes of adjacent cells. 10,000X

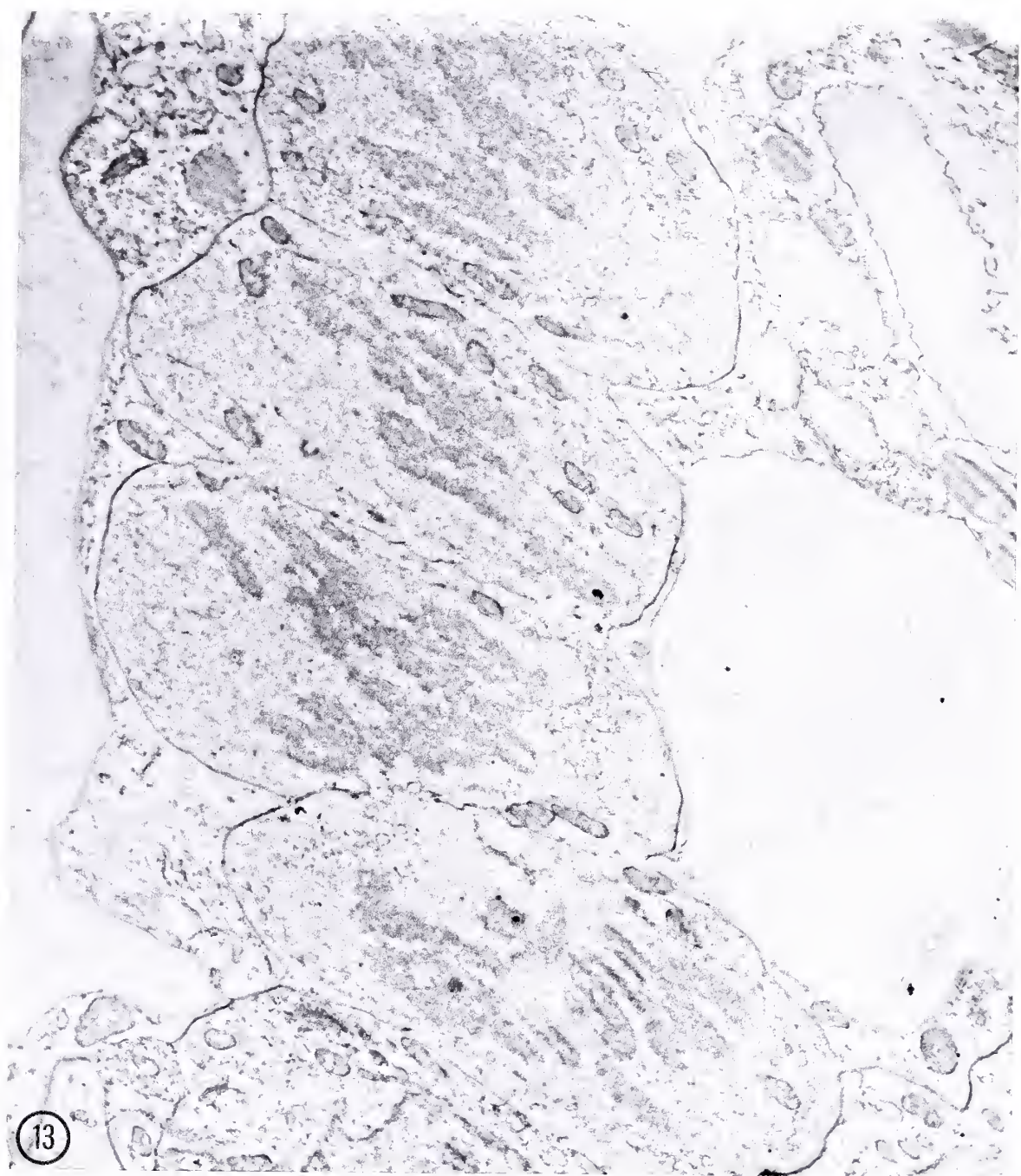


Fig. 14 Interstitial cells differentiating into cnidoblasts. Note that adjacent cells are connected by intercellular bridges. The bridges appear to be thickenings of the cell membrane situated perpendicularly to the membrane separating the two cells. Mitochondria, ribosomes, and vesicles are present in the cytoplasm.
12,000X

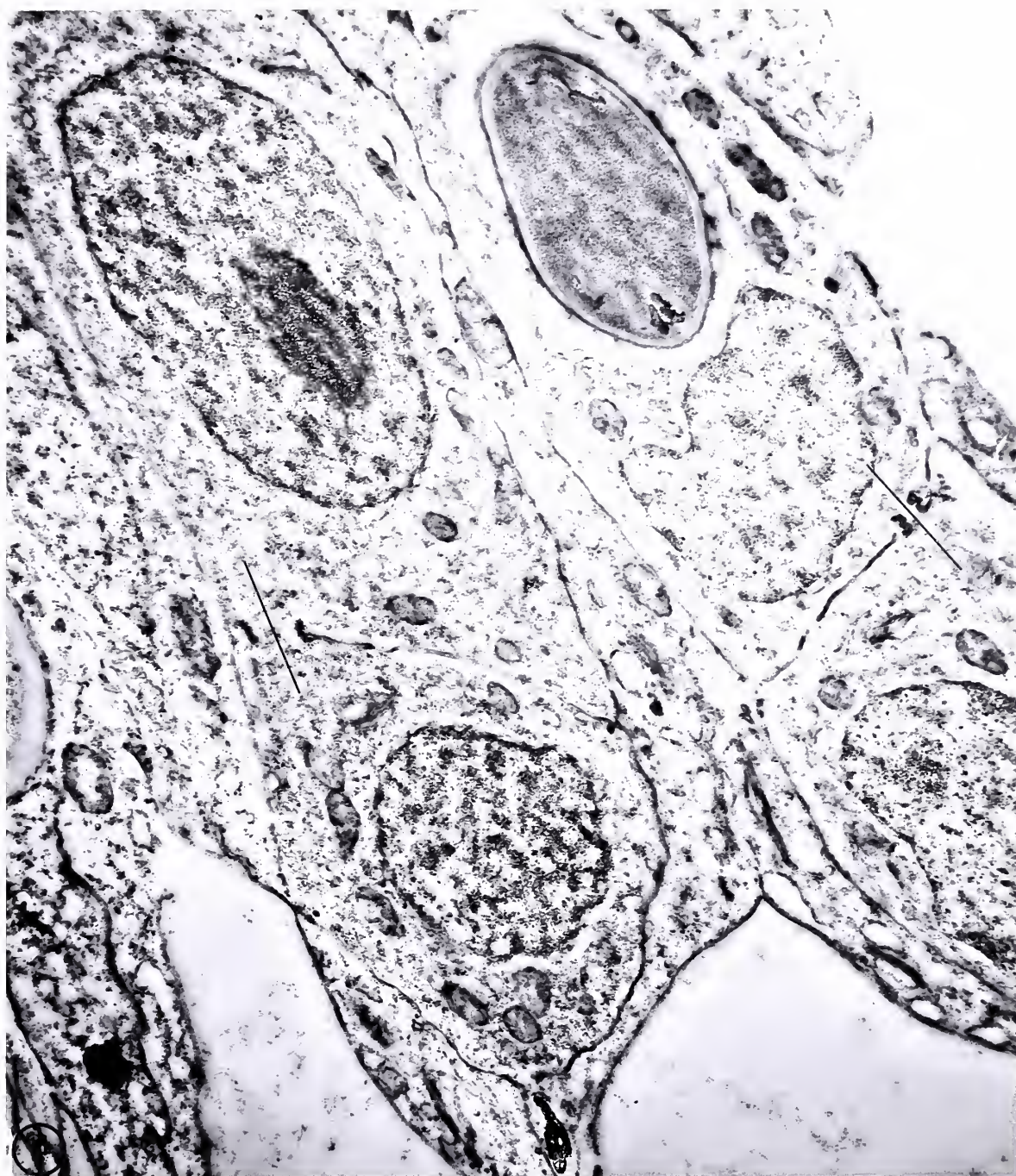


Fig. 15 Interstitial cell undergoing mitosis. Dense elongated chromosomes are present within the cytoplasm. Genes (arrows) are visible as dense transverse, paired striations in the chromosomes. No nuclear membrane is present. Note the large size of the chromosomes in relation to the mitochondria. 29,000X

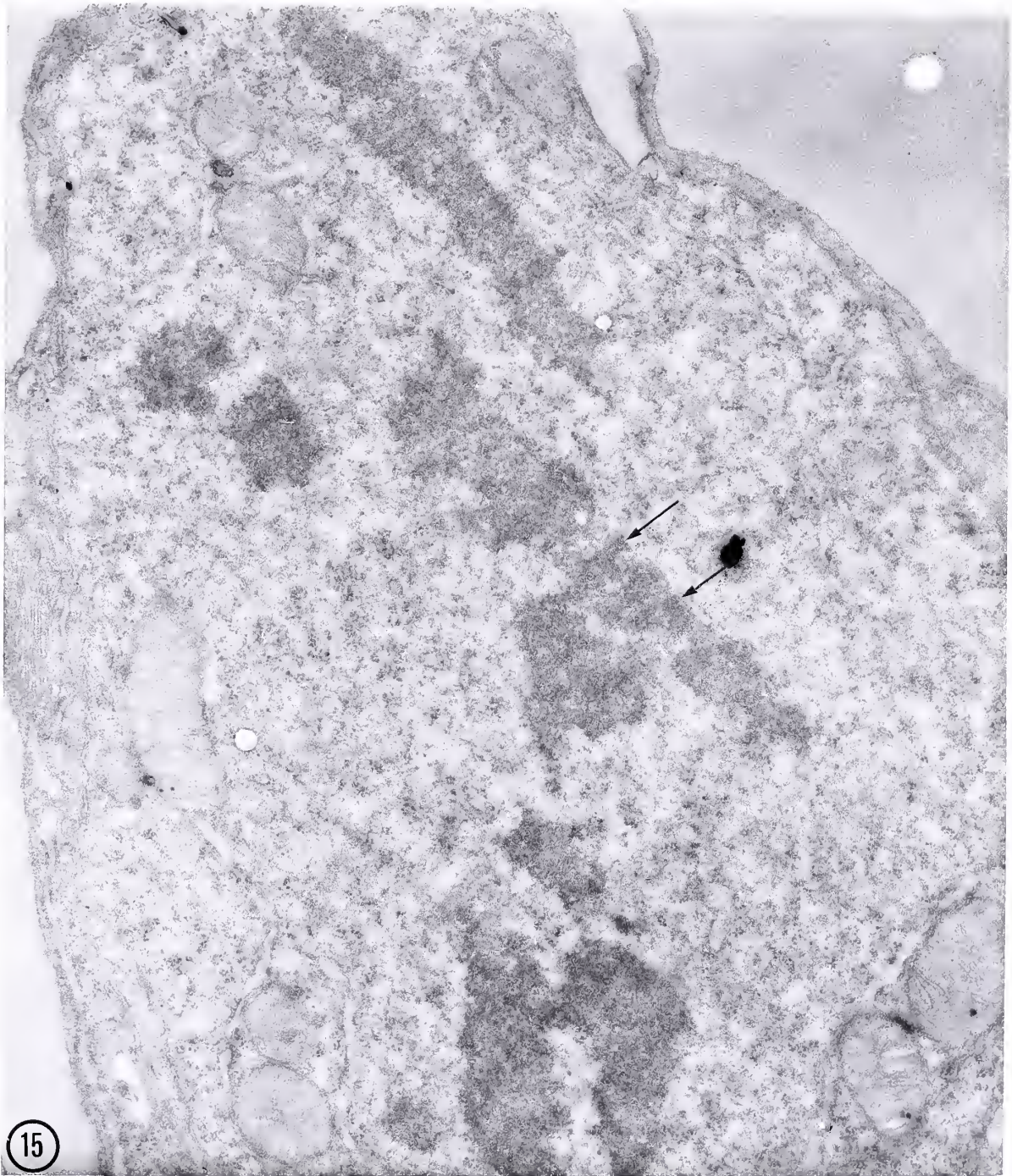


Fig. 16 Early stage in the development of a nematocyst. A Golgi apparatus surrounds the early nematocyst. Large, dilated vesicles (arrows) are situated immediately adjacent to the outer membrane of the nematocyst. Material elaborated by the Golgi apparatus may be deposited onto the nematocyst in this manner.

33,000X

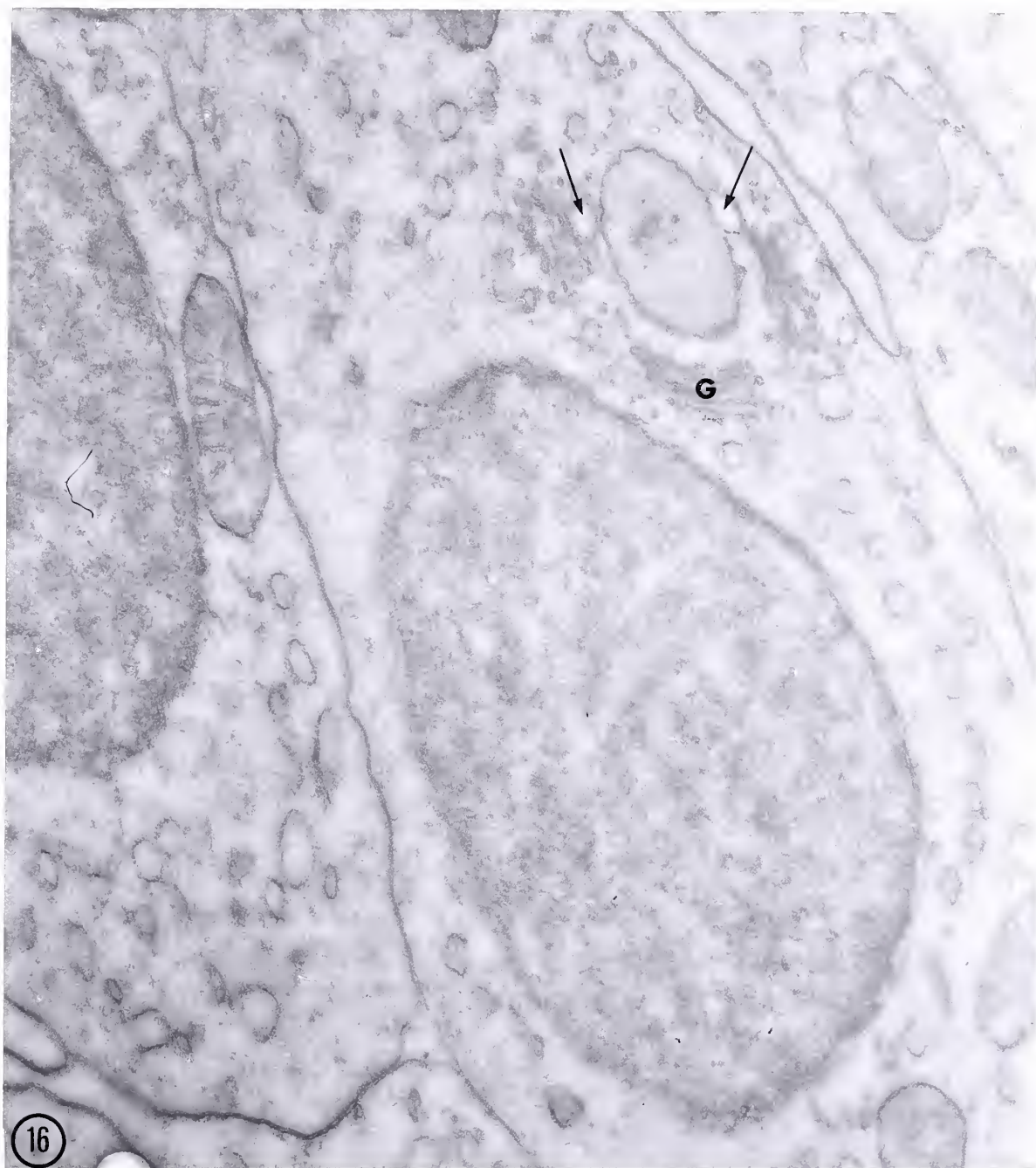


Fig. 17 Developing nematocyst. The small nematocyst is surrounded by small tubules and larger vesicles. The dense material within the nematocyst may be the precursor of the stylets and spines. Note the complex lamellar, rough-surfaced endoplasmic reticulum.

45,000X

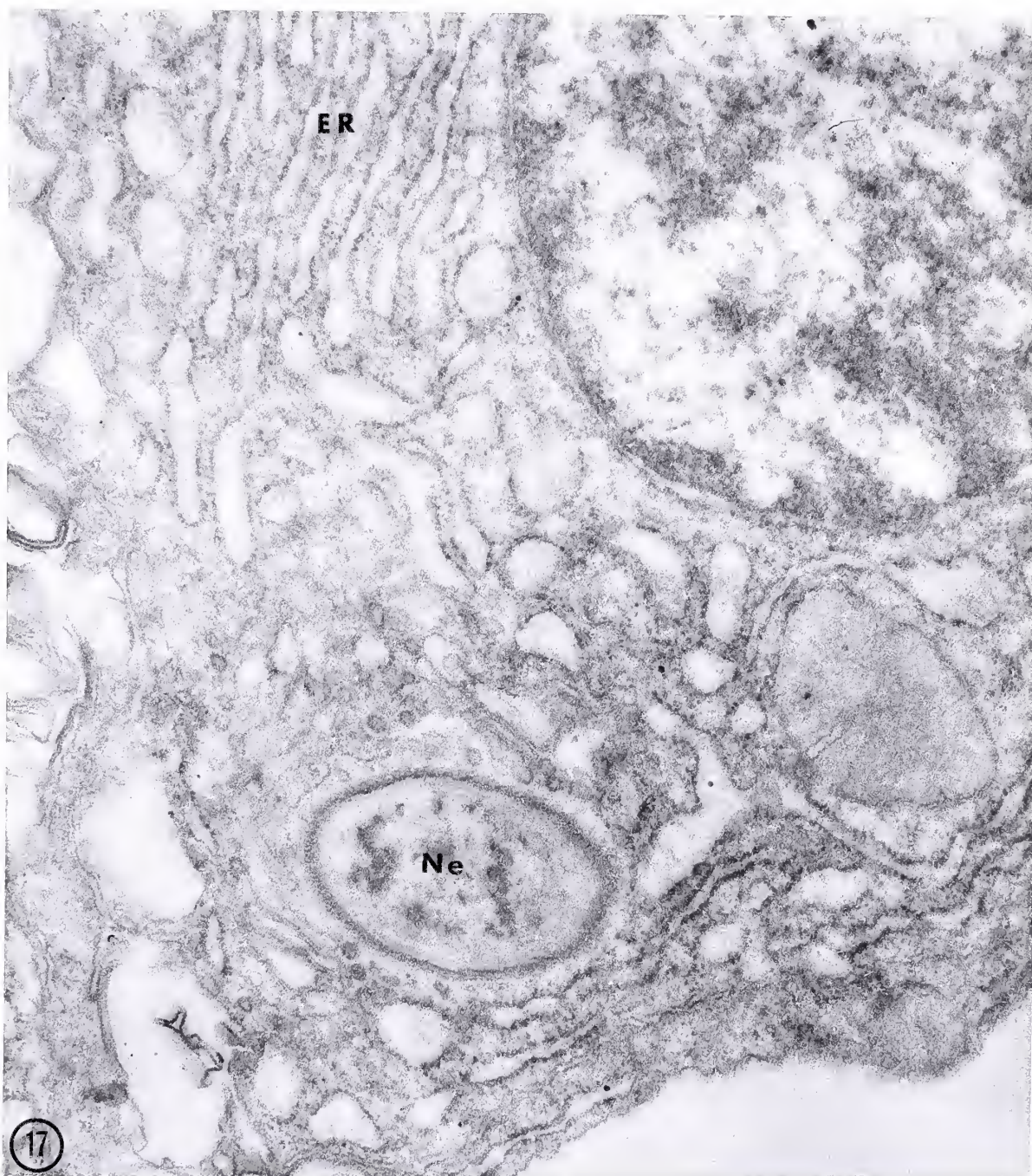


Fig. 18 Early stage in the development of a nematocyst. Many small vesicles (arrow) surround the nematocyst and are connected to the outer membrane of the nematocyst by a short tubular neck. Note the ribosomes attached to the outer membranes of the endoplasmic reticulum. The nucleus contains dense granular material. 120,000X

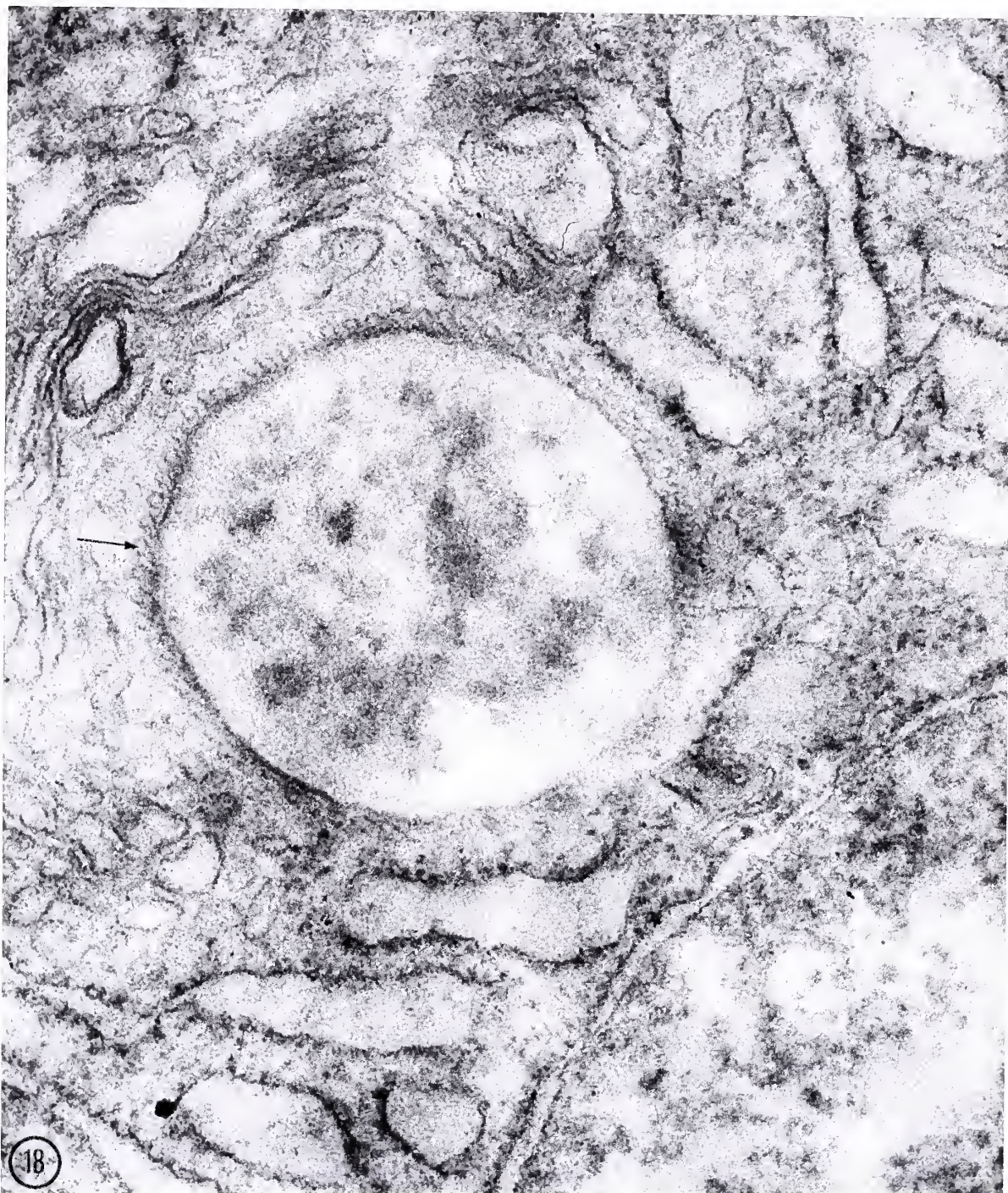


Fig. 19 Developing nematocyst surrounded by small vesicles. Note that some of the vesicles appear to be connected to the outer membrane of the nematocyst by a short tubule. Numerous Golgi vesicles are present in the cytoplasm of the cnidoblast. The endoplasmic reticulum is composed of rough-surfaced lamellae. 65,000X

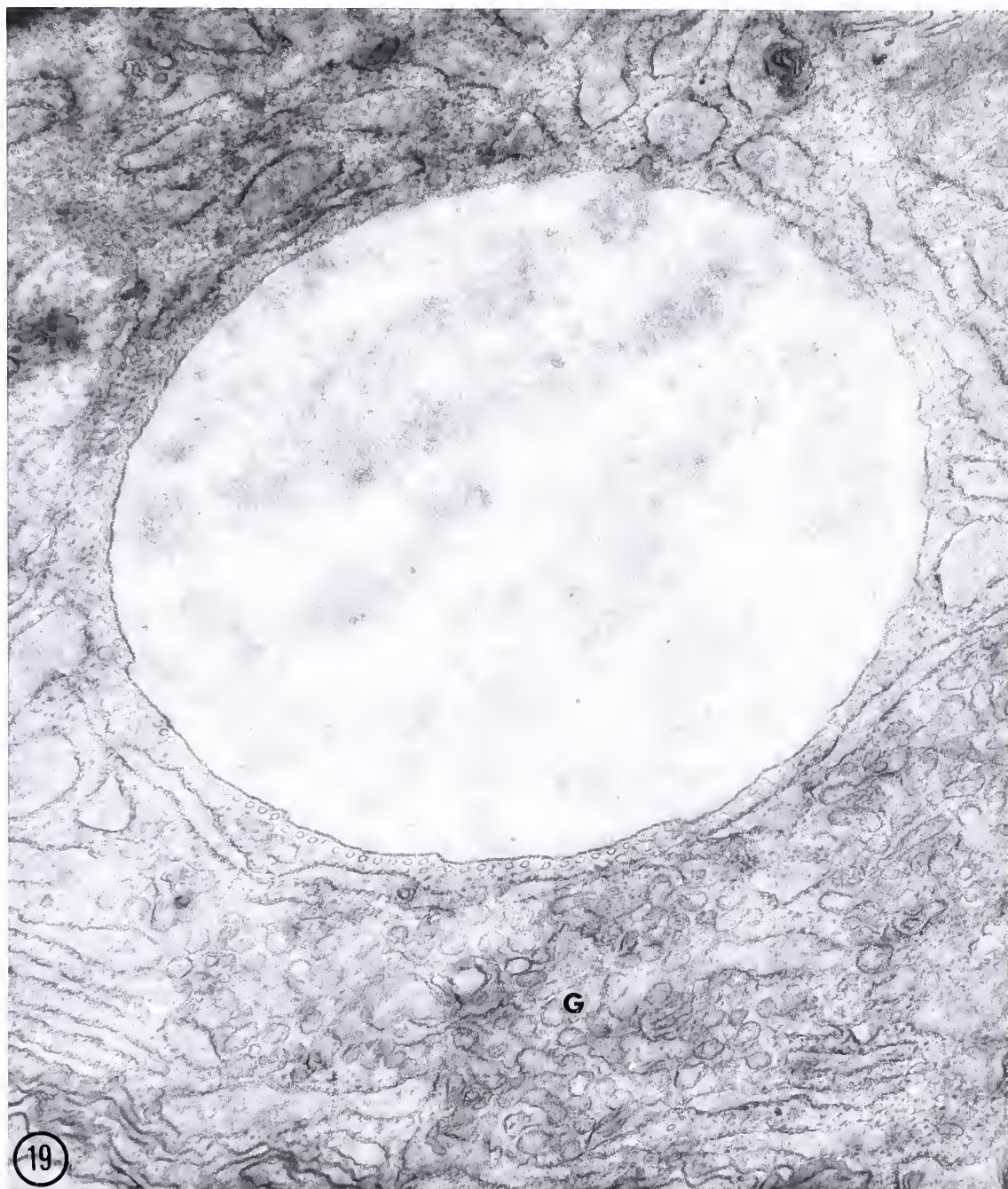


Fig. 20 Stage in the differentiation of a nematocyst. The endoplasmic reticulum is highly developed and an elaborate Golgi apparatus is situated at one side of the developing nematocyst. Dense granular material is present within the matrix of the nematocyst.
27,000X

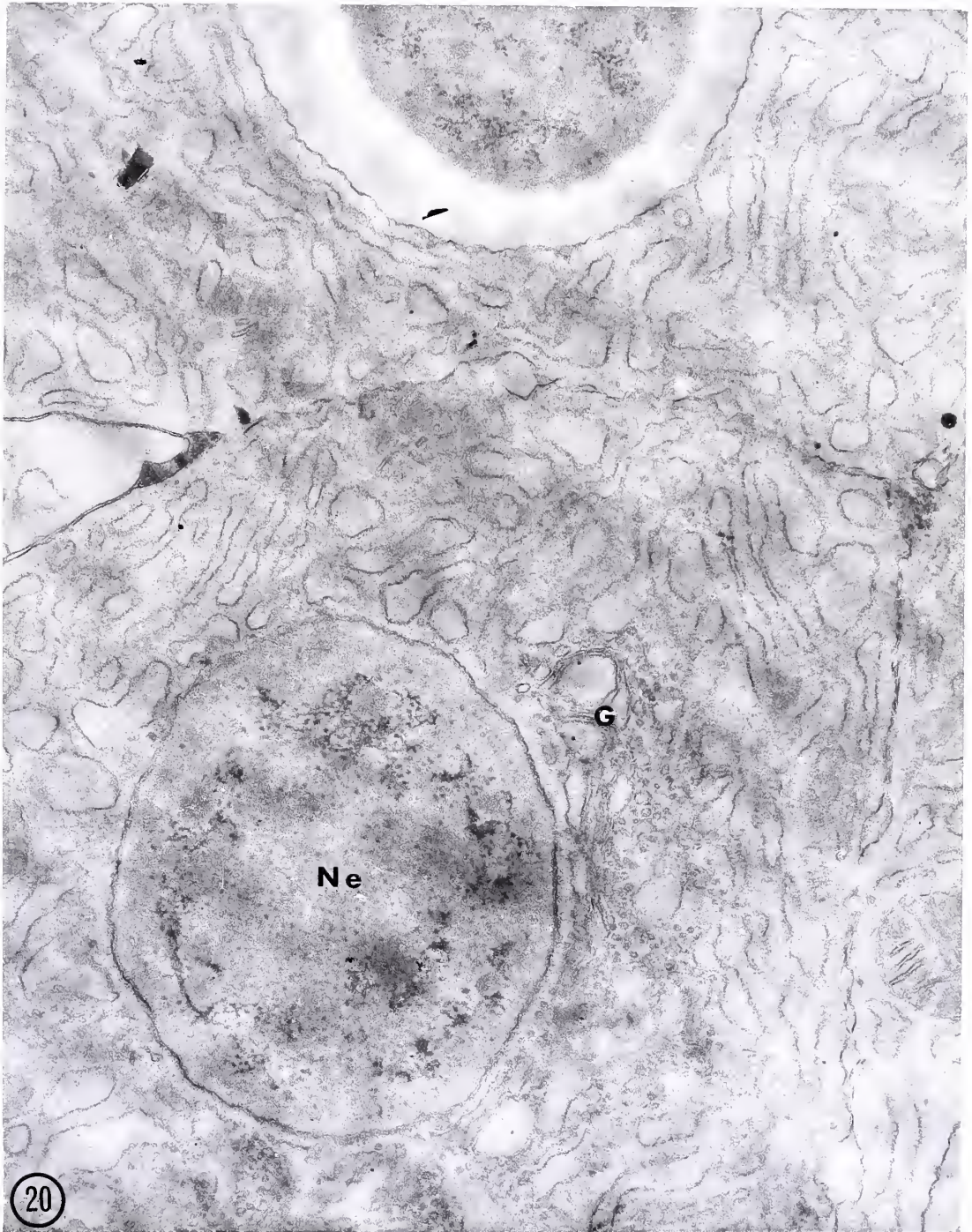


Fig. 21 Developing nematocyst. Note the capsule surrounding the granular matrix containing electron dense particles. The cytoplasm of the cnidoblast contains mitochondria and an elaborate endoplasmic reticulum. 27,000X

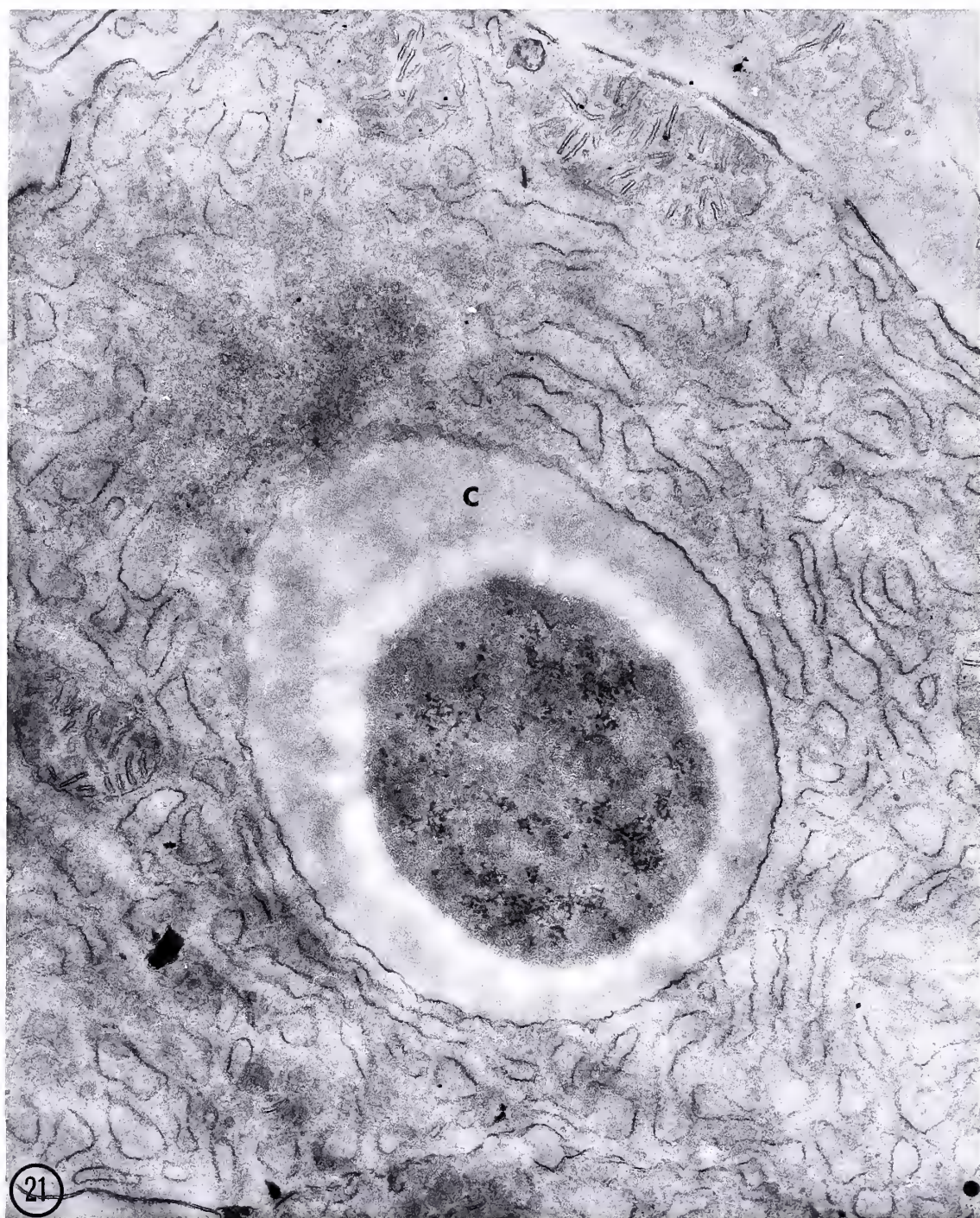


Fig. 22 Stage in the differentiation of a nematocyst (isorhiza). The endoplasmic reticulum is highly developed and the nucleolus is large and dense. Dense granular material is present within the matrix of the nematocyst. 27,000X



Fig. 23 Mature nematocyst (stenotele). Note the thread and invaginated capsular wall within the capsule. The operculum is situated at the apex of the nematocyst. The cytoplasm of the cnidoblast is undifferentiated containing a few vesicles and mitochondria. The nucleus is small and compressed to one side of the cell. 12,000X

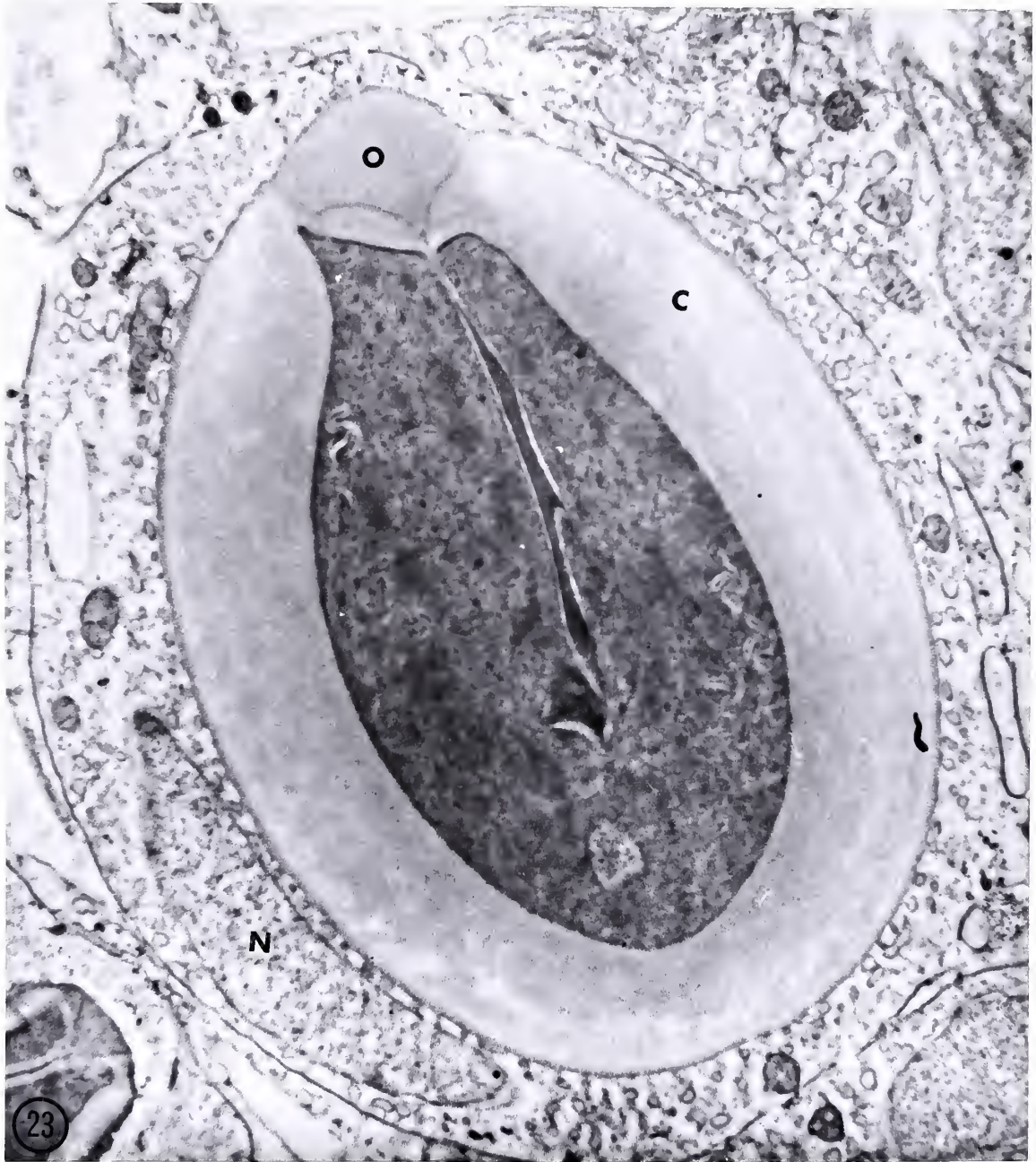


Fig. 24 Mature nematocyst (stenotele). The large dense stylets point toward the apex of the nematocyst and cover the smaller numerous spines. The invaginated capsular wall surrounds the stylets and spines. The thread is wound about in the basal portion of the nematocyst and bears the shape of a three bladed propeller. The homogeneous capsule surrounds all the intracapsular structures. 42,000X



Fig. 25 High magnification of the spines of a nematocyst. Note the large number of these structures closely packed within the invaginated capsular wall. 104,000X

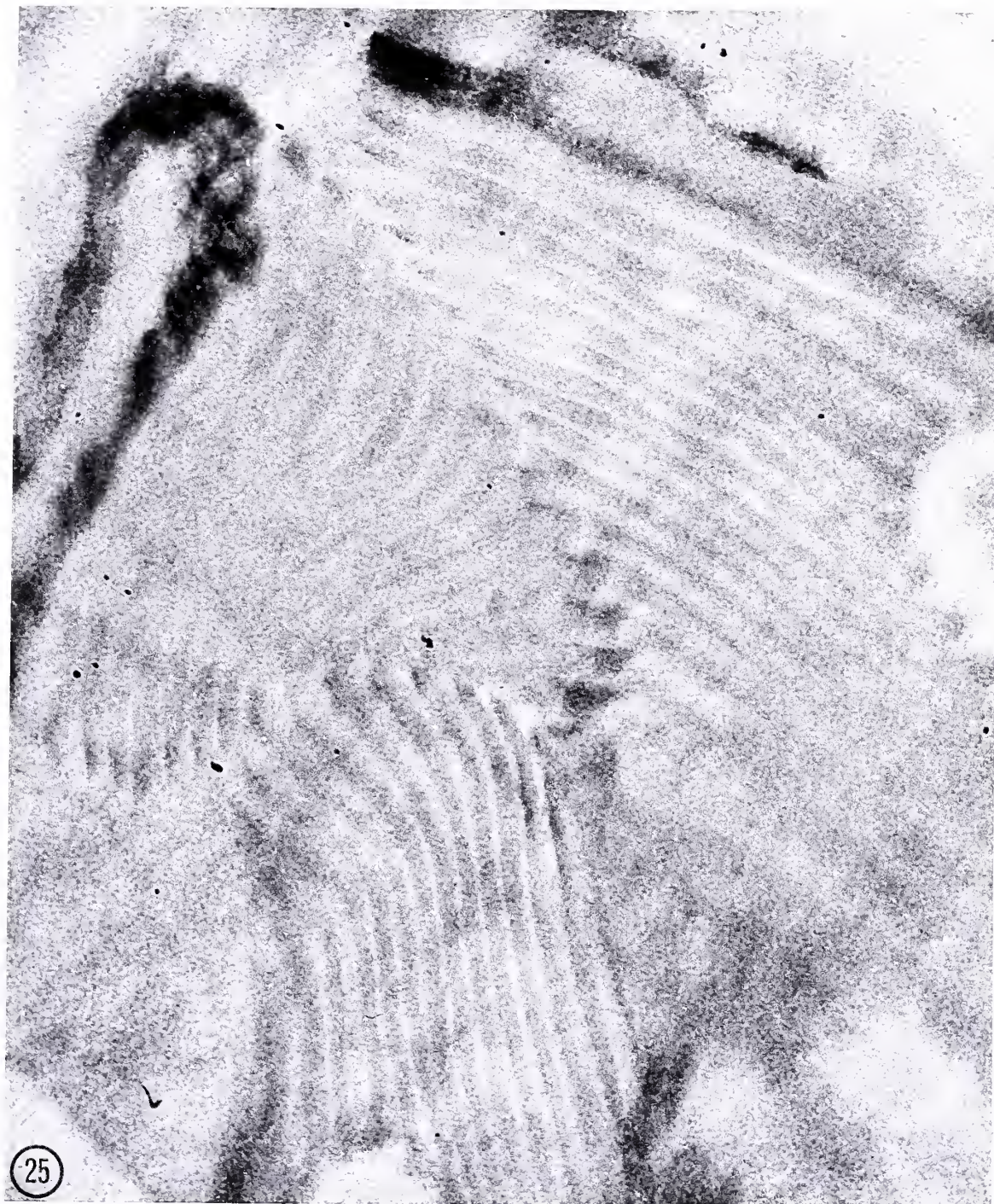


Fig. 26 High magnification of a nematocyst capsule and thread. The capsule is thick homogeneous and of low density. The thread in section bears the shape of a three bladed propeller. It consists of an outer layer of low density and a dense inner matrix. 104,000X

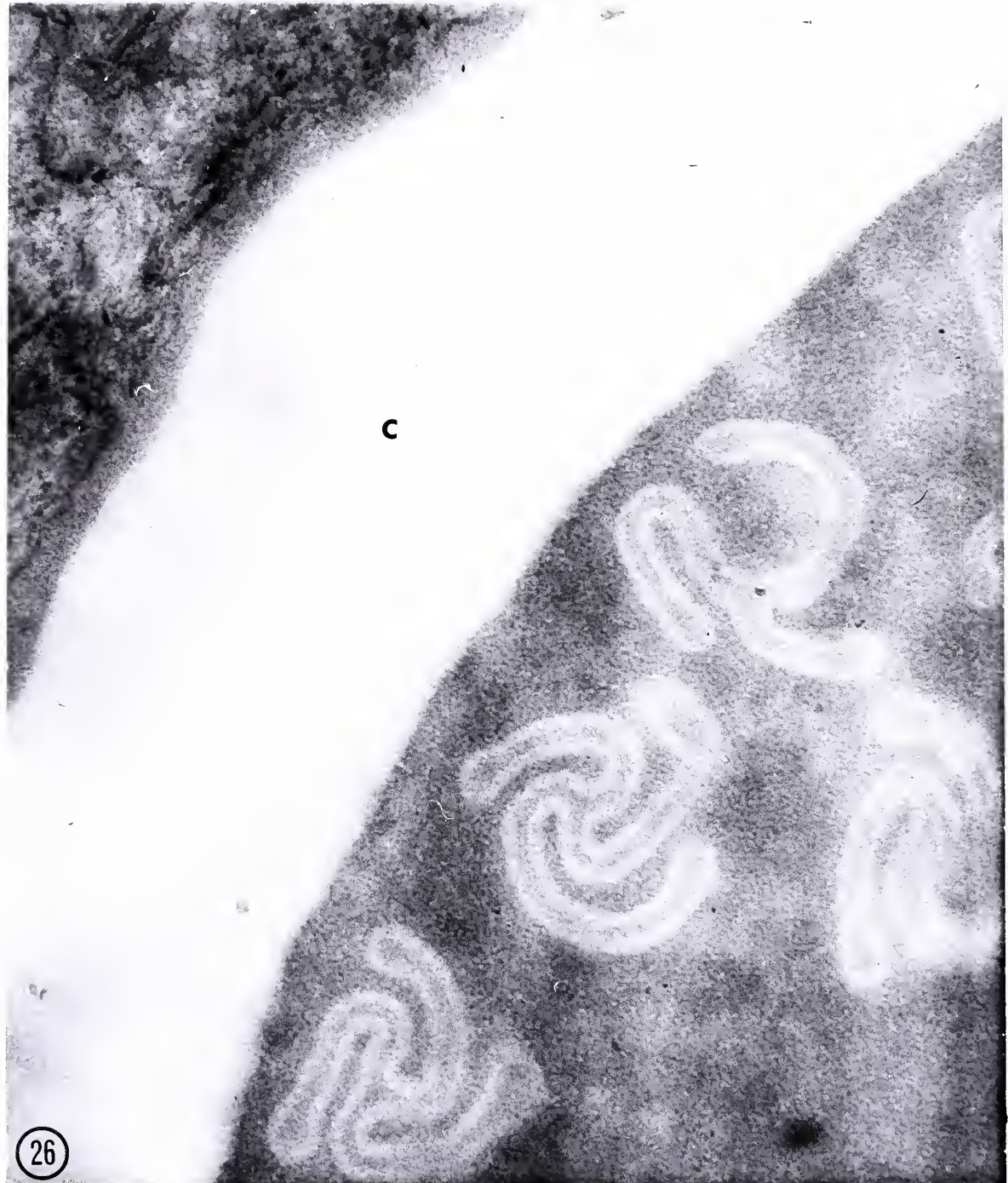


Fig. 27 Transverse section through the apex of the stylets. Note the density of these structures. The invaginated capsular wall surrounding the stylets possesses transverse densities situated at regular intervals. The three stylets surround a space through which the thread passes during discharge of the nematocyst. 74,000X



Fig. 28 Section through a nematocyst (desmoneme).
Note the small round shape of this type of nematocyst. The cytoplasm of the cnidoblast contains only a few vesicles and mitochondria. The nucleus is compressed to one side of the cell. 25,000X



Fig. 29 Cross section through three nematocysts (holotrichous isorhizas). The capsules of these nematocysts are thin. The large irregular threads are surrounded by invaginated capsular wall. The cytoplasm of the cnidoblasts contains mitochondria and smooth-surfaced vesicles. 25,000X

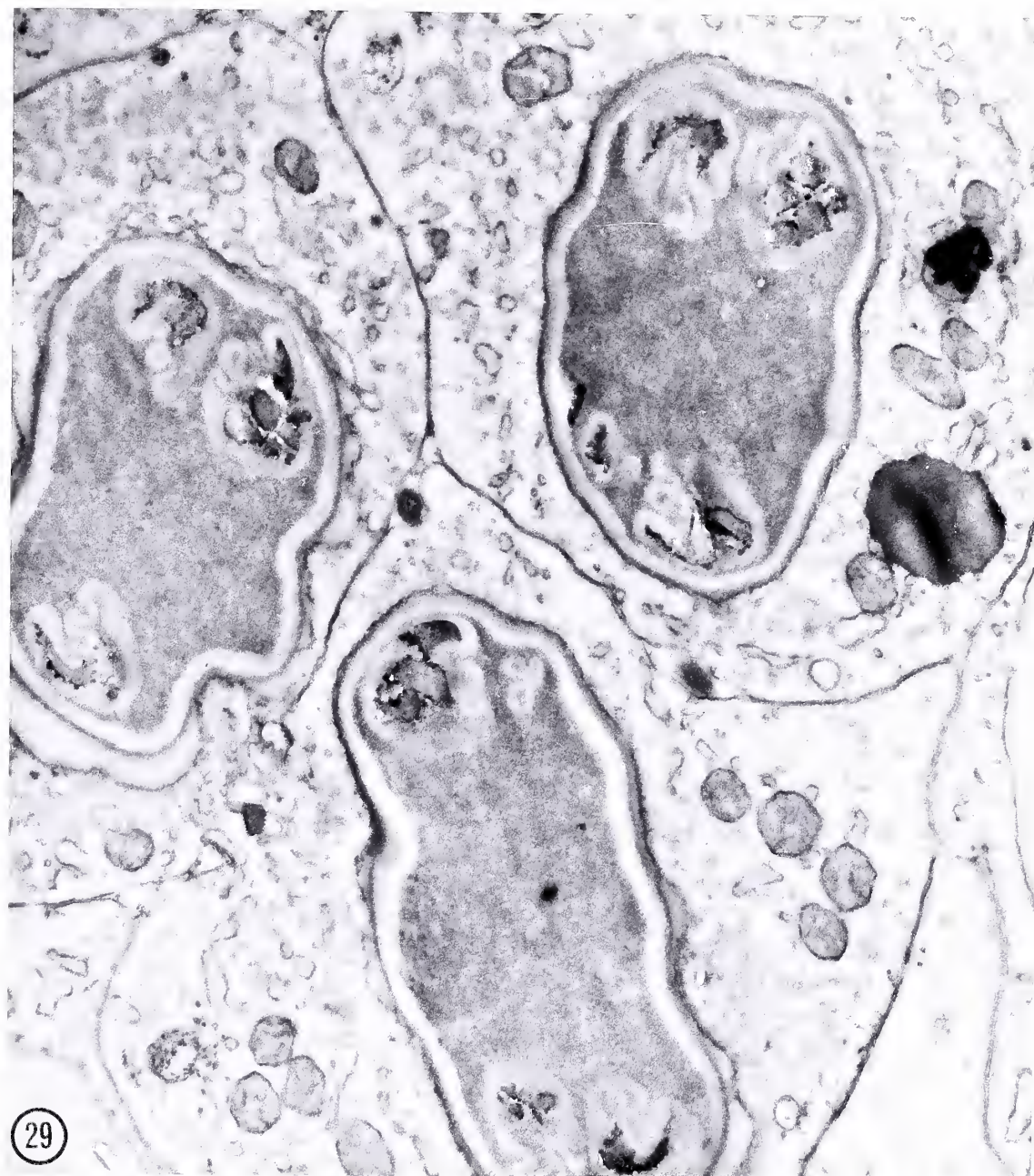


Fig. 30 Longitudinal section through a desmoneme and cnidocil. The supporting rods of the cnidoblast arise from the capsule of the nematocyst and are continuous with the larger supporting rods of the cnidocil. A large number of dense granules are present near the apex of the cnidoblast. 29,000X

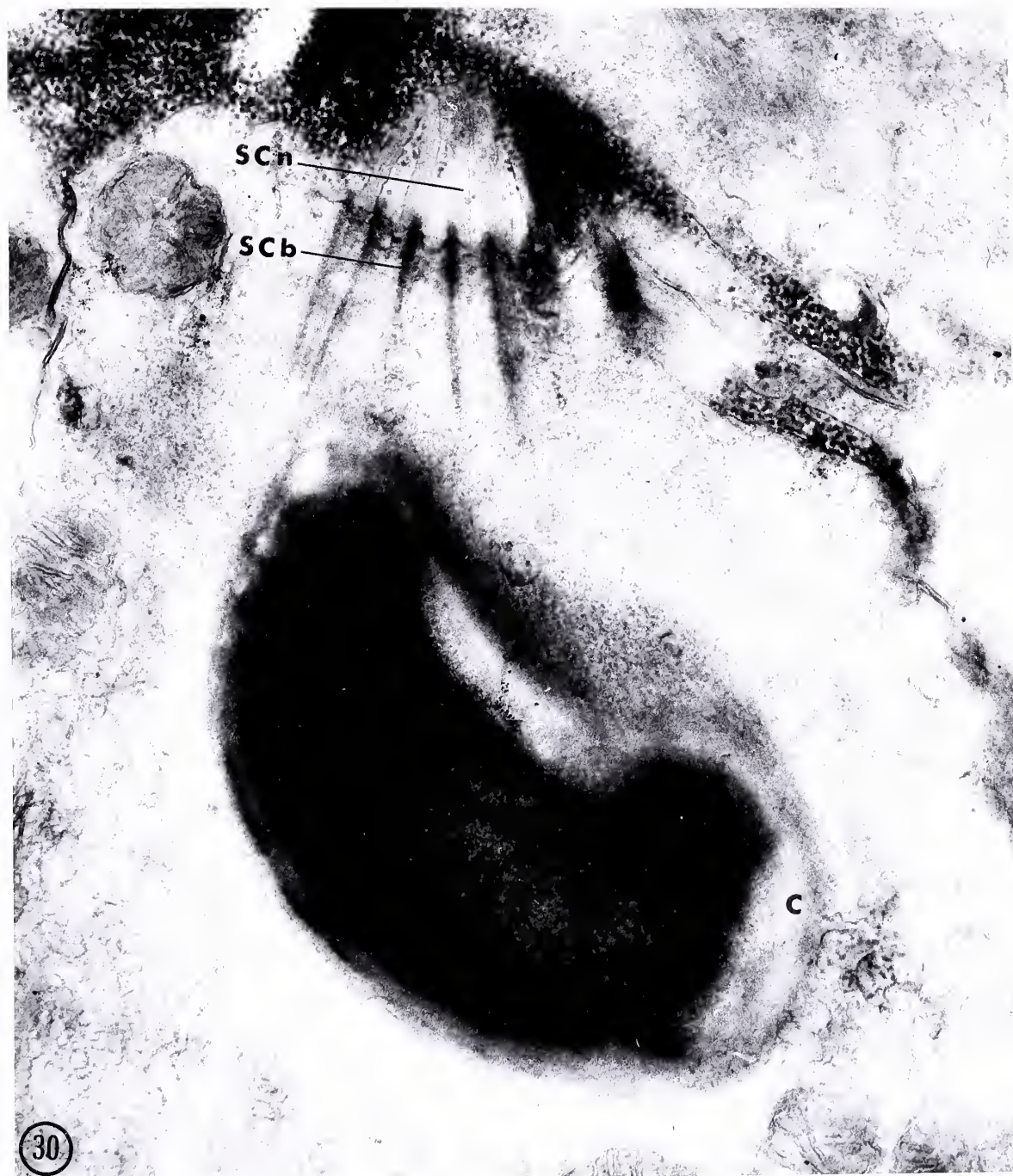


Fig. 31 Transverse section through a cnidocil.
A central core occupies the center of the cnidocil.
This structure is surrounded by the small dense
supporting rods of the cnidoblast. The large irregular
supporting rods of the cnidocil appear to arise
from the latter (arrows). Numerous dense granules
surround the cnidocil. 45,000X

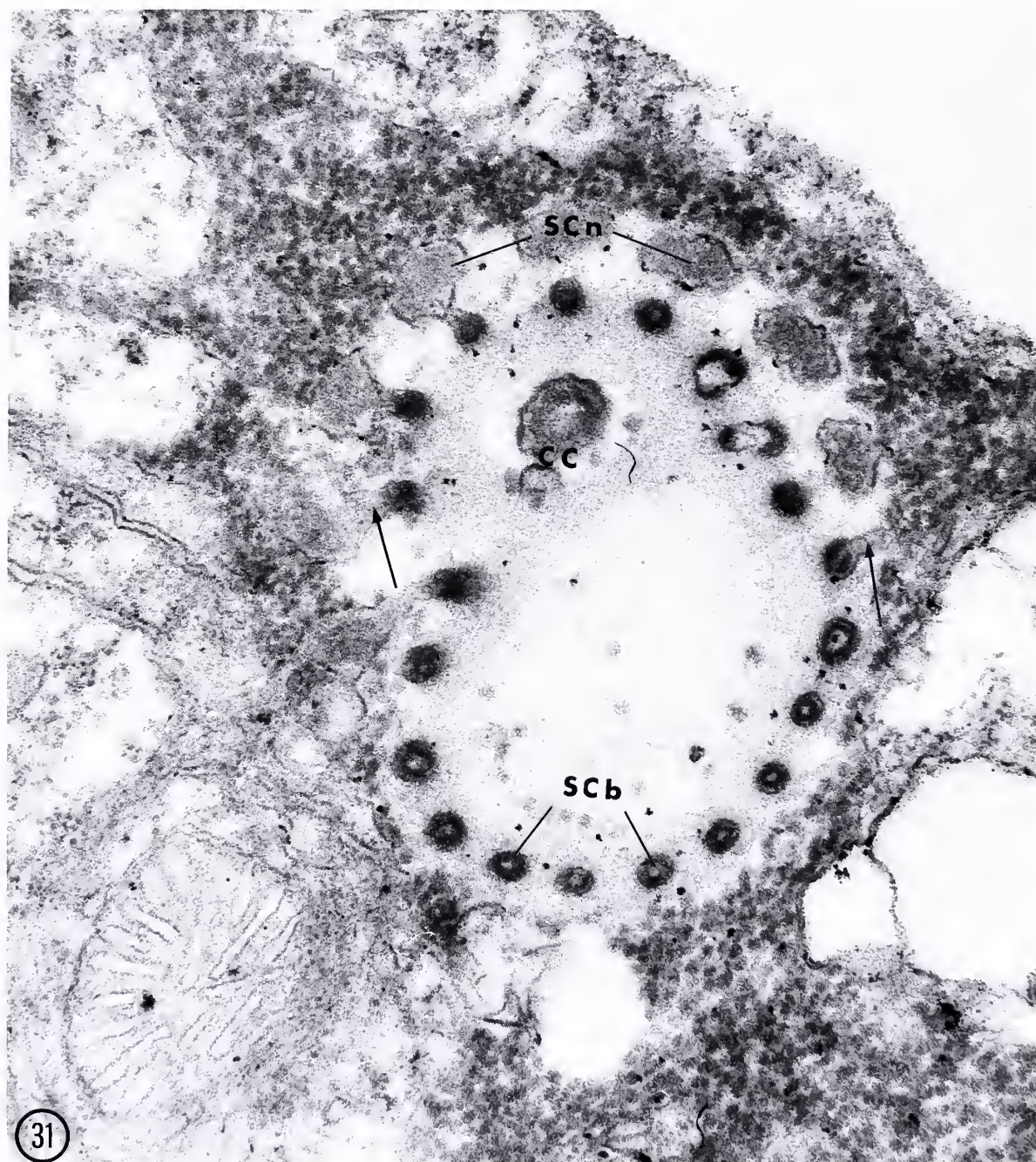


Fig. 32 Cross section through a cnidocil. The central core is separated from the supporting rods of the cnidocil by a band of finely granular material. An interstitial cell is located above the cnidocil.

46,000X

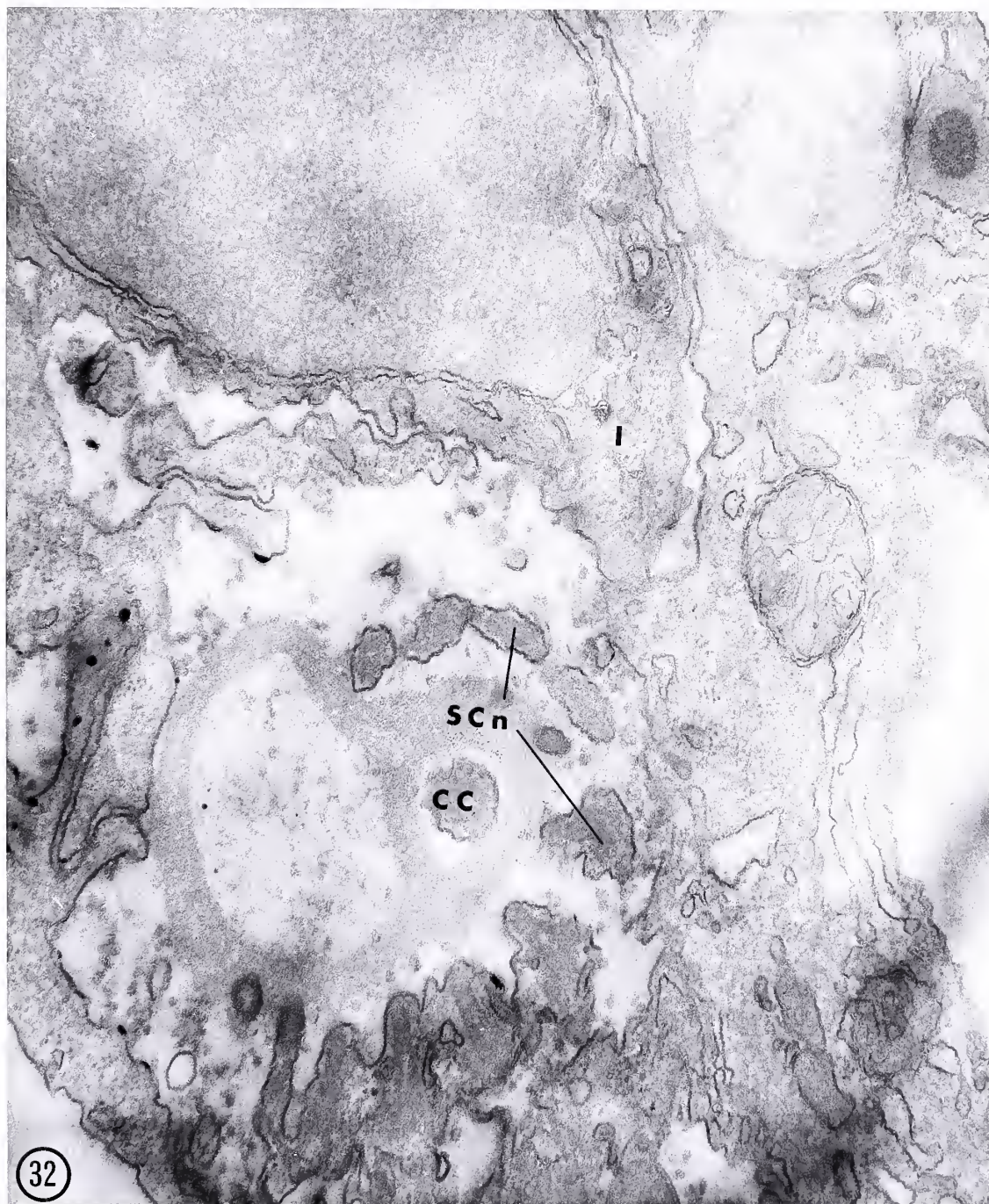


Fig. 33 Tangential section through the apex of a cnidocil. Note the presence of nine supporting rods of the cnidocil. surrounding a central core. The cnidocil is covered by the external protective layer of the epidermis. 46,000X



Chapter 9

Fine Structure of the Nervous System

This chapter presents a description of the fine structural details of the hydra nervous system which is composed of neurons and neurites or processes and which contains neurosecretory material. This problem was of particular interest since recent workers have not observed these cells with the electron microscope and have even denied the existence of a nervous system in the hydra (Hess, Cohen, and Robson, '57; Chapman and Tilney, '59; Slautterback and Fawcett, '59; Wood, '59; Hess, '61). Although the above mentioned workers have not identified at a fine structural level a cell type in the hydra that bears strict resemblance to mammalian neurons, it is impossible to deny the existence of a nervous system in hydra in view of the accumulated evidence from morphological (Mackie, 1881; Schneider, 1890; Zoja, 1892; Wolff, '04; Hadzi, '09; Marshall, '23; McConnell, '32; Hyman, '40; Mueller, '50; Semal-Van Ganssen, '52; Spangenberg and Ham, '60), physiological (Passano and McCullough, '62; Rushforth et al., '63), and histochemical (Chapter 3 and 4) studies.

Materials and Methods

Pieces of Hydra littoralis, especially from the hypostomal region, were fixed in 4% buffered glutaraldehyde followed by 1% buffered osmium tetroxide, embedded

in Maraglas, sectioned and examined in the manner described in Chapter 8.

Observations

During the study of the different cell types in various regions of hydra, elements of a well-developed, complicated and diffusely organized nervous system were found. This system was most concentrated in the hypostome and therefore, most of the present findings were gathered from careful study of this area. The nervous system is comprised of several cell types and their processes. The cell types are ganglion, neurosecretory, and sensory.

The small, elongated ganglion cells (Figs. 1 - 8) were situated at the base of epitheliomuscular cells just above their muscular processes. The nucleus was small and oval and was bounded by a nuclear envelope that contained numerous pores (Figs. 3 - 5). Nucleoli were not prominent or were absent. The cytoplasm of ganglion cells showed some variations in regard to ribosomal populations. Some cells contained numerous ribosomes lying free in the cytoplasm and unassociated with membranous components of the endoplasmic reticulum (Figs. 3 - 6). In these cells the ribosomes extended into the proximal portions of the processes and nothing comparable to an axon hillock was found. Other cells which grossly had the same shape and position were poor in ribosomes while

their processes contained none (Figs. 2 and 8). It is not known whether the distinctly different images represent two different cell types or the same cell type in different stages of maturation or function. Profiles of smooth or rough endoplasmic reticulum were present but not a prominent feature.

Two other morphological findings in the cytoplasm were considered significant. The first was the occurrence of microtubules only in the ribosome-rich ganglion cell (Figs. 3 - 5). These structures required very thin sections for their resolution and could be clearly delineated from profiles of endoplasmic reticulum. The microtubules were composed of a membranous envelope approximately 40\AA in diameter enclosing a space of 120\AA in diameter. These structures were situated in the cytoplasm in a direction parallel to the long axis of the neuron and extended out into the processes. At their central end they appeared to arise in the vicinity of nuclear pores (Figs. 3 and 4).

The other prominent feature of the cytoplasm common to both types of ganglion cells was an elaborate Golgi apparatus (Figs. 3, 6, 7, 8). In fact, some of the cells had two or three distinct Golgi regions (Figs. 7 and 8). This organelle was usually situated between the nucleus and a process. When it occurred in this morphological situation, its long axis was parallel to the long axis of the cell, extending from the nuclear region to the base

of a process (Fig. 8). When, on occasion, the organelle was unassociated with a process, its long axis was perpendicular to the long axis of the cell (Fig. 7). On some occasions the Golgi apparatus was located entirely within the base of a process.

Each Golgi apparatus was characteristically composed of flattened stacks of membrane-bound lamellae and small vesicles and large cisternae. In the Golgi that were oriented toward the process, the small vesicles appeared to arise by a pinching-off process from the ends of the lamellar stacks. These small vesicles appeared to contain a material of light and homogeneous density.

Mitochondria showing no unusual fine structural features were scattered irregularly in the hyaloplasm of the periparyon of neurons. However, they regularly occurred in relation to the elaborate Golgi apparatus: sometimes occurring parallel to the stacks of lamellae but more often occurring at the ends of the lamellae where small vesicles were in the process of budding off (Fig. 8).

Processes (neurites) extended from the perikaryon of ganglion cells in between other cell types. In many cases these processes were so tortuous that at best they could be followed for only 10 to 15 microns and were of irregular diameter, containing bulbous enlargements in some areas (Fig. 2). The neurites contained microtubules (Fig. 9), small vesicles containing a material of light

density, and interspersed mitochondria. It should be noted that the processes were lined only by the plasma membrane of the cell. Nothing comparable to a myelin sheath or surrounding glial supporting cells were ever found.

Terminations of neurons were difficult to recognize or indeed find because of the tortuosity of the fibers. However, it appeared that at least some of the fibers ended at the base of cnidoblasts (Fig. 10) or near the processes of some epitheliomuscular cells (Fig. 11). When this occurred, the ending appeared as a bulbous cluster containing a few vesicles and small mitochondria. No morphological specializations of surface contacts comparable to synaptic regions in higher forms were found. On the other hand, some of the processes appeared to be in direct contact with extracellular spaces and the mesogleal region (see below).

Neurosecretory cells appeared entirely different from ganglion cells (Figs. 12 and 13). Although these cells were rare, they could be easily recognized by the presence of a large number of cytoplasmic granules. The granules were small (about 500\AA in diameter) and were composed of a dense homogeneous substance. These granules occurred as membrane-bound accumulations within neurosecretory cells and in close relation to the Golgi apparatus. In these cases, the granules were located in

the dilated ends of the parallel lamellae of the Golgi and in small vesicles immediately adjacent to these structures as though having bud off from the lamellae (Figs. 14 and 15). In some cases, the entire cytoplasm and processes of neurosecretory cells were filled with these granules (Fig. 14). In most cases, the granules in the processes appeared free of their membranous envelope (Fig. 16).

The neurites of neurosecretory cells terminated in an extremely complicated manner. In some cases, a bulbous enlargement was present which constricted to narrow channels only 500\AA in diameter (Fig. 17). Bulbous enlargements as well as constrictions frequently were filled with granules. In addition, it often appeared that the processes were directly continuous through discontinuities in the plasma membrane with the extracellular space which also contained similar granules (Figs. 13 and 17). This space in turn was directly continuous with the mesoglea where similar granules were also found.

Sensory cells were small and elongated structures situated between and near the apical surface of epithelial or digestive cells. The cytoplasm contained a well-developed Golgi apparatus and numerous vesicles. Two types of sensory cells could be distinguished on the basis of apical specializations. One type (Figs. 18 and 19) possessed a structure that resembled a modified cilium in that it

contained a limiting membrane, a central core, and nine small peripheral rods. The intracytoplasmic portion of this structure was surrounded by a dilated membranous sac. The second type of cell possessed a blunted or bulbous apex which protruded to the surface between adjacent cells. The opposite pole of these cells was blunted, drawn into a cytoplasmic process, or was directly adjacent to a ganglion cell.

Discussion

It is perhaps surprising that previous investigators have been unable to identify any component of the hydra nervous system with the electron microscope. Previous studies (see Introduction) provide ample evidence for the existence of a nervous system in hydra. On the other hand, it should be realized that the hydra possesses one of the most primitive nervous systems of all the metazoa. It is doubtful if the morphological criteria for the identification of mammalian neurons can be applied to the nervous system of hydra. Furthermore, nerve cells are not as numerous as other cell types and sections obtained through the cell body and processes are indeed fortuitous.

The nervous system as seen with the electron microscope corresponds closely to previous descriptions made with the light microscope. Certain criteria, based on findings with the light microscope, may be used in

identifying the nervous system at a fine structural level. The two most important are position and shape. The ganglion cells lie above the muscular processes of the epidermal and digestive cells and are most abundant in the hypostome. They are easily distinguished on the basis of their processes and cytoplasmic specializations from interstitial cells which are situated between epitheliomuscular cells, predominantly in the growth region which has few neurons. Ganglion cells are bipolar or multipolar and their neurites extend above the mesoglea and the bases of epidermal and digestive cells. The concentration of these cells in the hypostome suggests the beginnings of an organized central nervous system.

Sensory cells which contain a specialized apical organelle are interspersed between other cells of the epidermis or gastrodermis. They terminate below the surface in a bulb or send a specialized process (modified cilium) to the exterior while the basal pole usually possesses a process. Presumably, the ganglion and sensory cells are concerned with the transmission of impulses.

The most striking feature of the hydra nervous system is the presence of neurosecretory cells which are most abundant in the hypostome. These cells were characterized on the basis of the definition established by the International Symposium on Neurosecretion (Scharrer, '62) (in particular, the presence of neurosecretory granules)

and also on the fact that they do not form synapses with other neurons or effector organs. In hydra, the granule-laden cells did not appear to form synapses but terminated blindly between other cell types and at times seemed to liberate their contents into the extracellular spaces. In this regard, the intercellular spaces correspond to the circulatory system of higher animals. The ganglion cells, on the other hand, contained many small vesicles which also might have a neurosecretory function. This cell, however, terminated on cnidoblasts, epitheliomuscular cells, and other ganglion cells. This observation suggests that these cells are responsible for transmission and the vesicles may play a chemical role in this process. This cell, therefore, may possess both nervous and endocrine properties and such a possibility should not be ruled out.

Granules observed within the neurosecretory cell were present within the cell body and the processes. A granule identical to the intracellular type was present in the intercellular spaces and mesoglea. Histochemical evidence suggests that at least some of the granular material may be neurosecretory. The localizations of acetylcholinesterase, epinephrine, norepinephrine, and 5-hydroxytryptamine were presented in Chapters 3 and 4. These substances were present as tiny granules within neurons and their processes. In addition, accumulations

of granular material staining for these substances were observed at the bases of cnidoblasts and near muscular processes of epitheliomuscular cells. These localizations were identical to the areas of greatest concentration of the 500\AA granules and also the vesicles of ganglion cells.

Slautterback and Fawcett ('59) believe that the granules represent glycogen. The 500\AA granules, however, are most numerous in the epidermis and mesoglea and absent in the peripheral cytoplasm of digestive cells. Yoder ('26) and Burnett ('59) identified glycogen in the digestive cells but found very little in the mesoglea and epidermis. Smaller granules, $150\text{--}300\text{\AA}$ in diameter, have been observed in greatest numbers in digestive cells, especially near the surface, which corresponds to the histochemical localizations of glycogen. The small granules were rare, although present, in the epidermis and mesoglea. Lead hydroxide, which stains glycogen, stained the membrane-bound accumulations of granules at the apical end of digestive cells but this method only stained few of the mesogleal and epidermal intercellular granules. On the basis of these studies it is felt that probably some of the granules, especially the smaller ones corresponding to those within digestive cells, are glycogen but others represent neurosecretory material elaborated by neurons and liberated into intercellular spaces.

The neurosecretory granules appear to be elaborated

by the Golgi apparatus as in practically all other neurosecretory cells (see Bern et al., '62). Vesicles and granules were found in greatest abundance near the Golgi apparatus. In some cases granules were present in the dilated ends of Golgi lamellae and within vesicles of the Golgi apparatus.

The structure of the nervous system as seen with the electron microscope could shed some light on the physiology and function of the coelenterate nervous system. There has long been a controversy as to whether the nervous system is synaptic or continuous (see Hyman, '40, '59). On the other hand, it is generally agreed that conduction can occur equally well in all directions. Structurally, the nervous system was found to be composed of separate neurons, but typical synapses were not observed. Instead, terminations of neurites consisted of bulbous enlargements containing a few vesicles; others contained massive accumulations of extracellular granules believed to represent neurosecretory material. Because of the lack of structural polarization, it is probable that impulses could pass in either direction across nerve terminals. Thus, in view of the latter suggestion, the nervous system could be composed of separate units and still conduct impulses in any direction over widespread areas. In this regard, Passano and McCullough ('62) using microelectrodes obtained rhythmically occurring potentials originating

from many foci which they believed to be nerve action potentials.

Cnidoblasts appear to be in intimate contact with the nervous system of hydra. Numerous investigators have described a relationship between nerves and cnidoblasts (Korotneff, 1876; Jickeli, 1882; Chun, 1881; Lendenfeld, 1887; Murbock, 1893; Kepner, '43; Spangenberg and Ham, '60). with the electron microscope this relation is even more intimate than supposed. Ganglion cells, sensory cells, neurites, and granules were found to be in association with cnidoblasts. Of constant occurrence was the presence of a clump of granules situated next to the cnidoblast. The suggestion (Chapter 5) that the nervous system plays a significant role in nematocyst discharge is made even more binding by these findings.

A final matter concerns the existence and function of neurosecretory material. Histochemical (Chapter 3 and 4), physiological (Chapter 7), and pharmacological (Chapter 7) studies suggested that the hydra possessed neurosecretory material and that these substances play a key role in the growth processes of this animal. The present studies have established a morphological basis for neurosecretion since nerve cells contained membranous vesicles and dense granules which appeared to be elaborated by the Golgi apparatus. Presumably, one or both of these structures contain the substances which play a key role in

regulating growth, differentiation, and the anatomical form of this animal.

Summary

1. The fine structure of the hydra nervous system is presented in this chapter. Several cell types and their processes comprise the nervous system. The cell types are ganglion, neurosecretory, and sensory.
2. The ganglion cells were small and elongated and situated at the base of epitheliomuscular cells just above their muscular processes. Some cells contained numerous ribosomes lying free in the cytoplasm while others were poor in ribosomes. One or more elaborate Golgi complexes were situated between the nucleus and a process. Each Golgi apparatus was composed of flattened stacks of membrane-bound lamellae, large cisternae, and an abundance of small vesicles. Microtubules were situated in the cytoplasm and appeared to arise in the vicinity of nuclear pores and to extend out into the processes. Processes (neurites) extended from the perikaryon of ganglion cells in between other cell types.
3. Neurosecretory cells were characterized by 500⁰Å granules within the cytoplasm and processes. Granules were located in the dilated ends of the parallel lamellae of the Golgi and in small vesicles immediately adjacent to these structures as though having bud off from the lamellae.
4. Sensory cells were small and elongated cells situated between and near the apical surface of epithelial or

digestive cells.

5. Morphologically, hydra nerve cells are capable of neurosecretion, since membranous vesicles and dense granules appeared to be elaborated by the Golgi apparatus. It is suggested that the substances which regulate or control growth, differentiation, and the anatomical form of the animal (Chapter 7) are contained within one or both of these structures.

Abbreviations for Electron Micrographs

C	capsule	Mu	mucous granule
CC	central core	MV	microvillus
EFL	external fibrillar layer	N	nucleus
EPL	external protective layer	NC	nerve cell
ER	endoplasmic reticulum	Ne	nematocyst
F	lipid droplet	Nl	nucleolus
FV	food vacuole	NP	nuclear pore
G	Golgi apparatus	NS	neurosecretory granule
GC	ganglion cell	O	operculum
Gl	gland cell	P	nerve cell process (neurite)
Gly	glycogen	PV	pinocytotic vesicle
I	interstitial cell	S	secretory droplet
ICS	intracellular space	SC	sensory cell
ICW	invaginated capsular wall	SCb	supporting rod of cnidoblast
M	mitochondrion	SCn	supporting rod of cnidocil
Me	mesoglea	Sp	spines
Mf	myofibrils	St	stylets
MG	mucous gland cell	T	thread (filament)
MP	muscular process		
MT	microtubule		

fig. 1 Low magnification of the epidermis. Note the epitheliomuscular cell containing a large nucleus and dense nucleolus. A small ganglion cell is located above the muscular processes. A sensory cell is embedded in the apical portion of the epitheliomuscular cell. 4500X

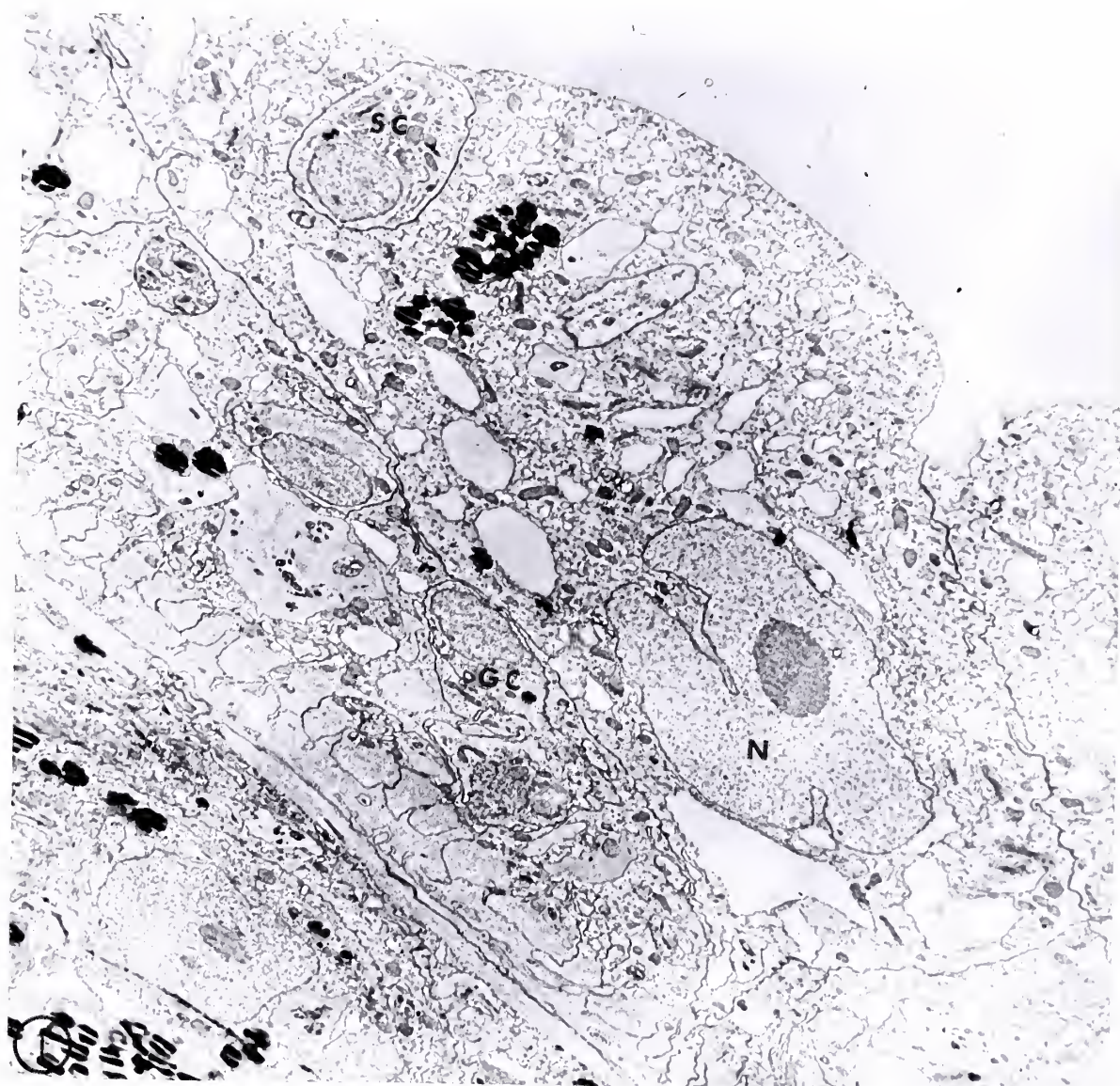


Fig. 2 Ganglion cell and process (neurite). The cell lies above the muscular process of an epitheliomuscular cell. A Golgi apparatus is situated between the nucleus and the process. The process contains a few tubules and mitochondria and terminates as a bulbous enlargement. This cell possesses few ribosomes. 21,000X

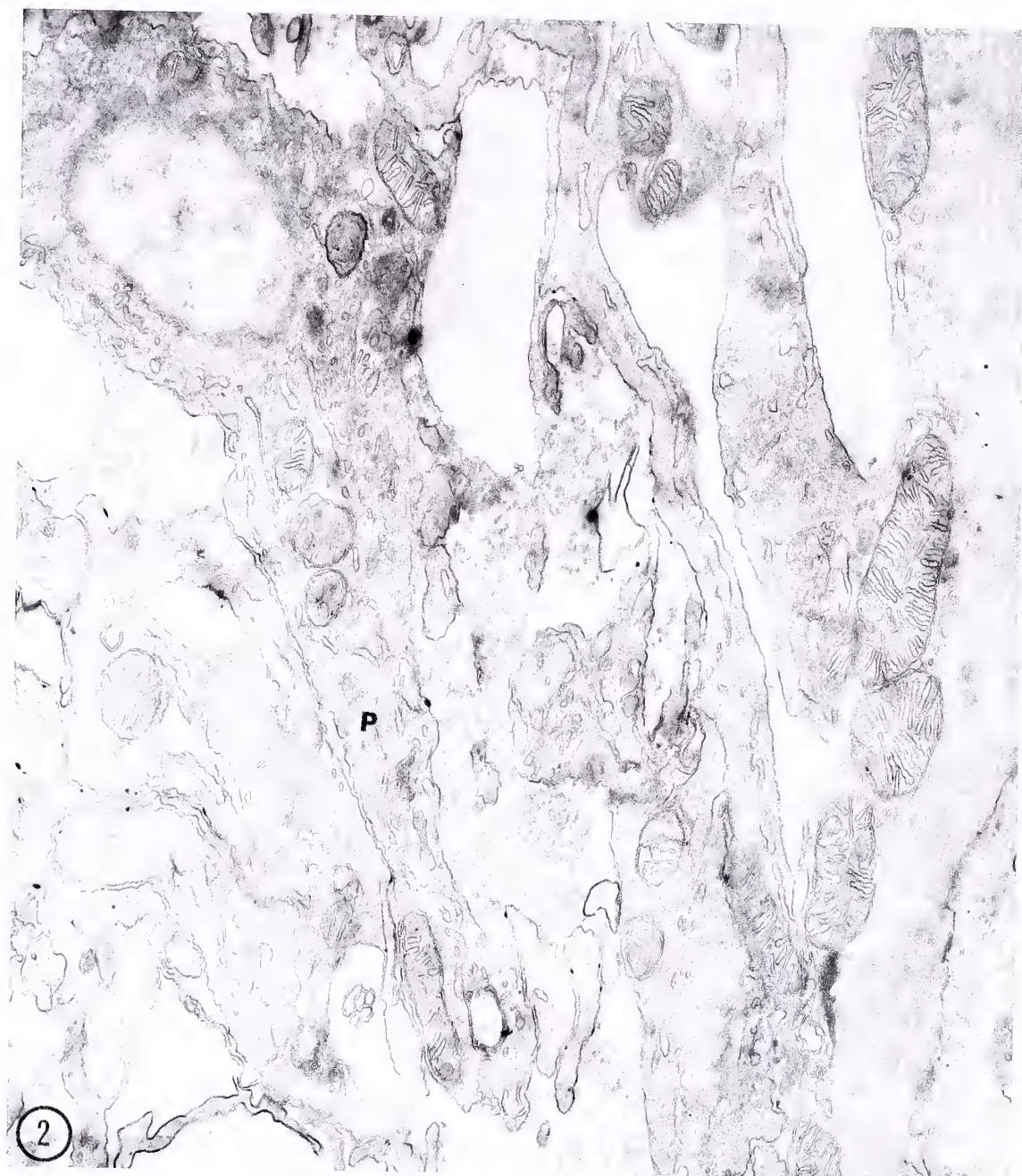


Fig. 3 Ganglion cell. The section passes tangentially through the nuclear membrane which possesses numerous pores. Microtubules are present in the cytoplasm and some are connected to the nuclear pores (arrows). The cytoplasm also contains a Golgi apparatus, ribosomes, and mitochondria. 29,000X

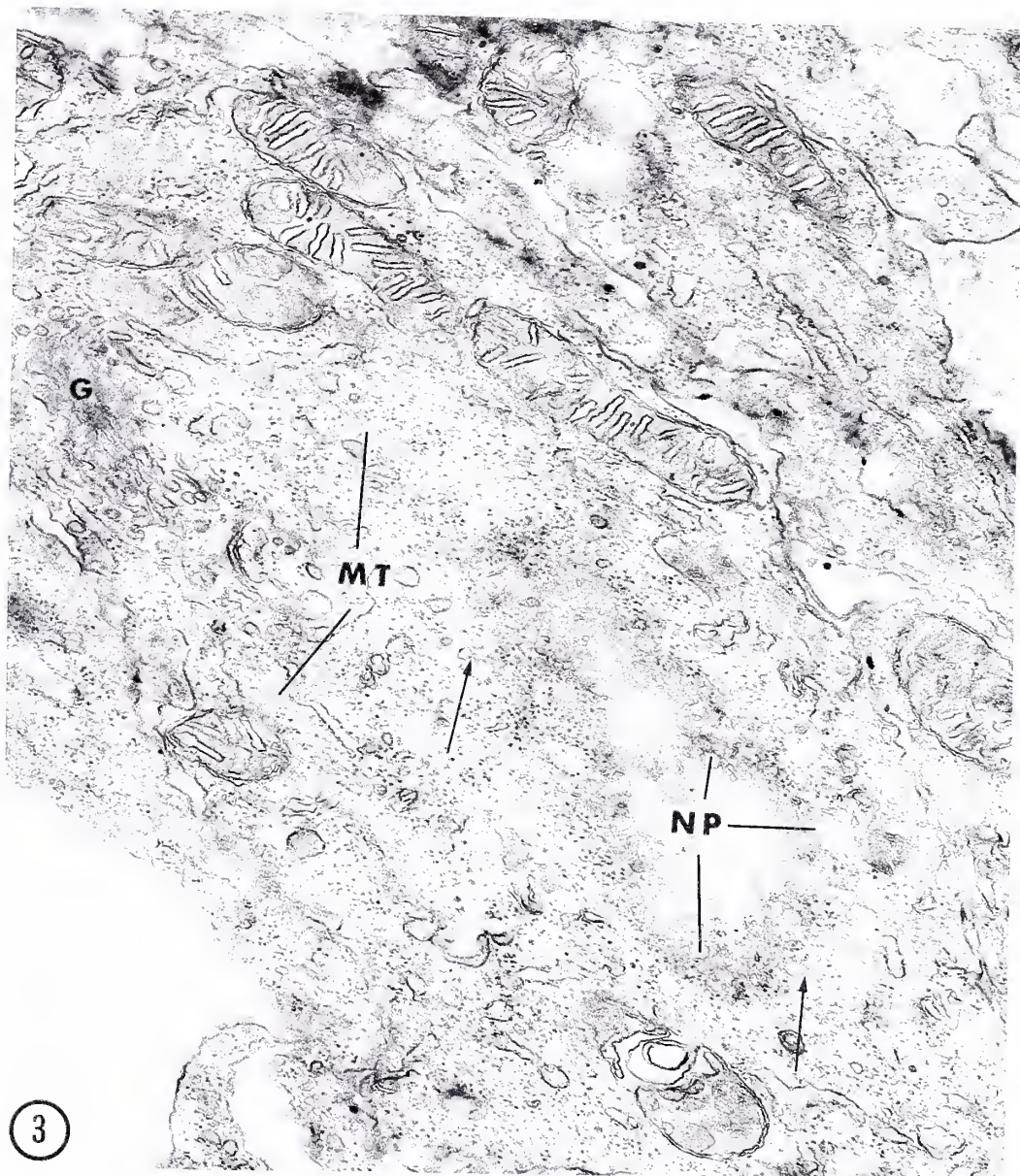


fig. 4 Tangential section through the nucleus of
a ganglion cell. Note that some of the microtubules
appear to arise from the nuclear pores (arrows).

45,000X

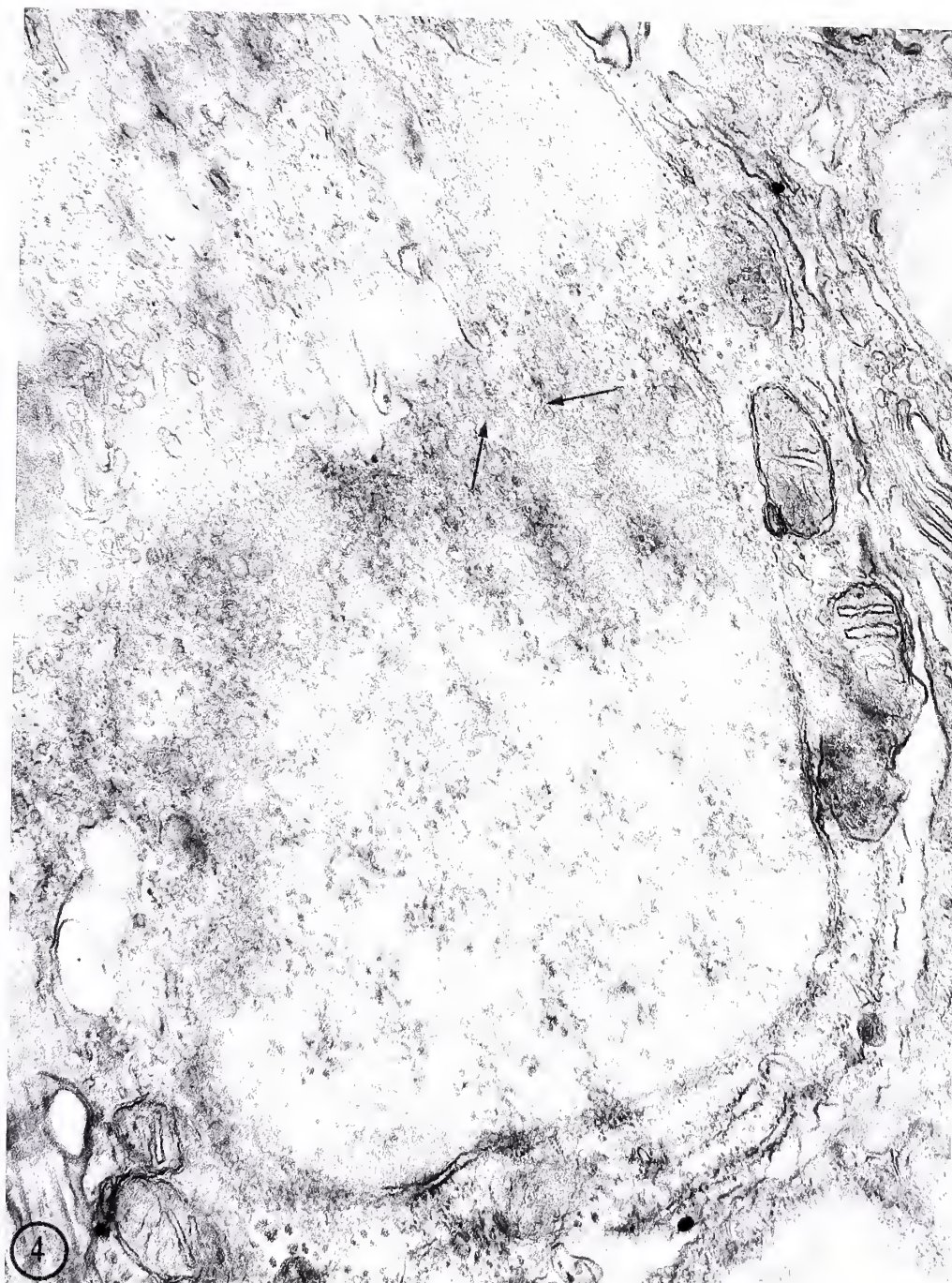
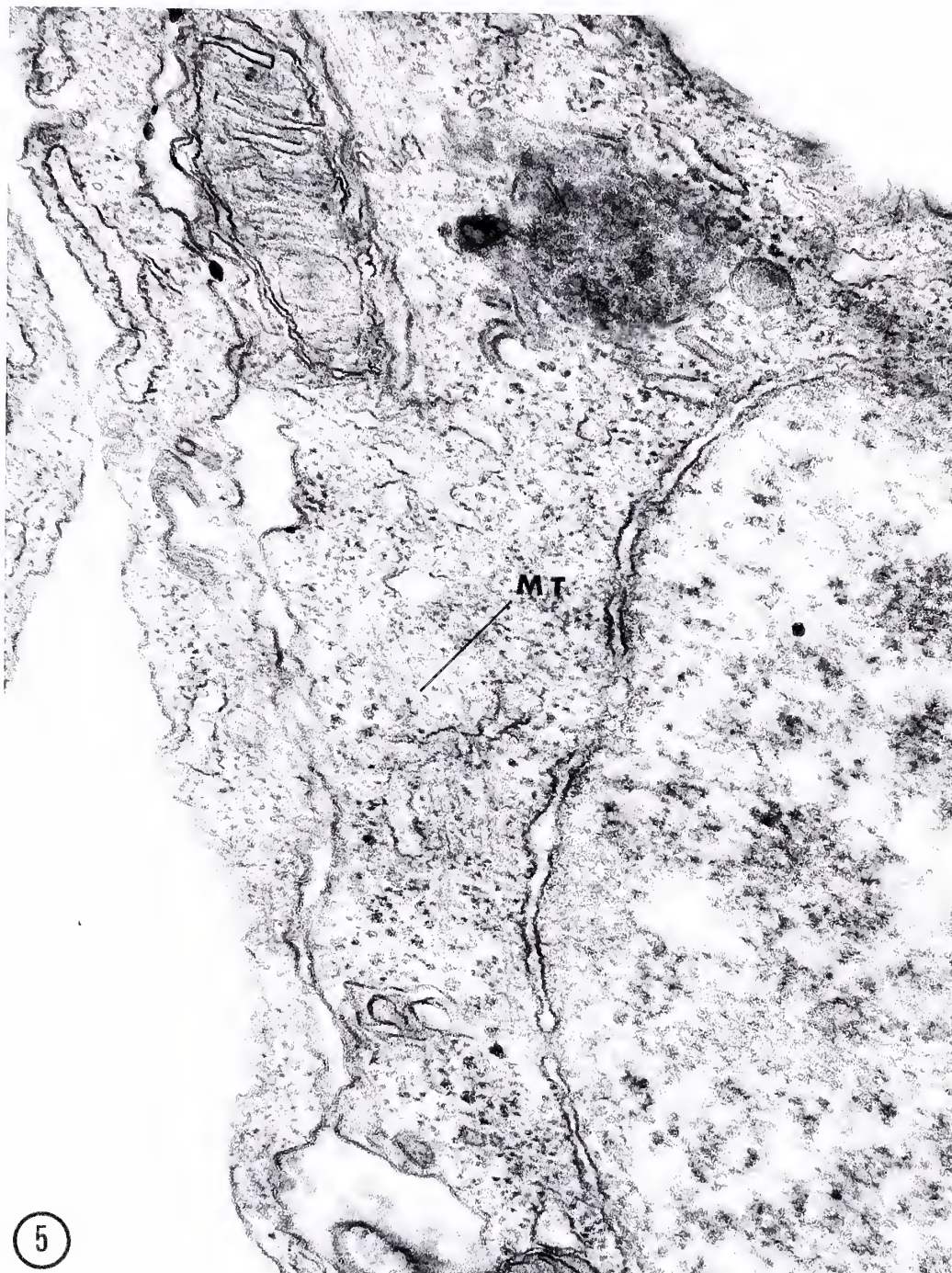


Fig. 5 Nucleus and cytoplasm of a ganglion cell.

The nucleus is surrounded by a double membrane containing fenestrations. Microtubules are present in the cytoplasm extending longitudinally to the long axis of the cell. Many ribosomes and a mitochondrion are also present in the cytoplasm. Note the membrane bound accumulation of dense granular material.

65,000X



5

Fig. 6 Ganglion cell. Note the complex Golgi apparatus situated near the nucleus. The Golgi is composed of lamellae and numerous vesicles. The vesicles extend into the surrounding cytoplasm. Numerous ribosomes and a few large membranous lamellae are present in the cytoplasm.

45,000X

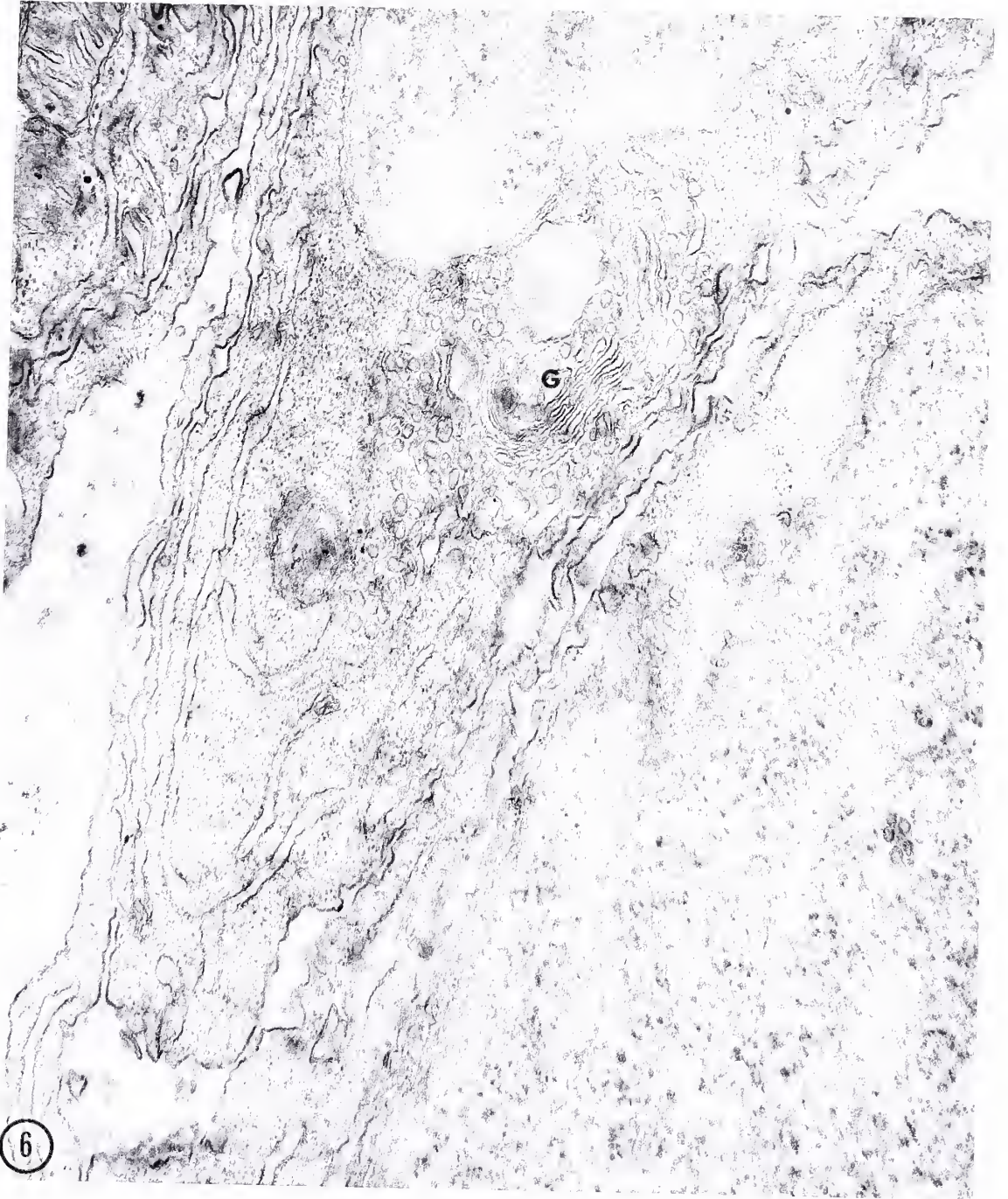


Fig. 7 Ganglion cell containing three Golgi complexes. Note that two Golgis are situated between the nucleus and cell processes. The Golgi complexes are composed of flattened stacks of lamellae and small vesicles. Mitochondria and ribosomes are also present in the cytoplasm. 45,000X

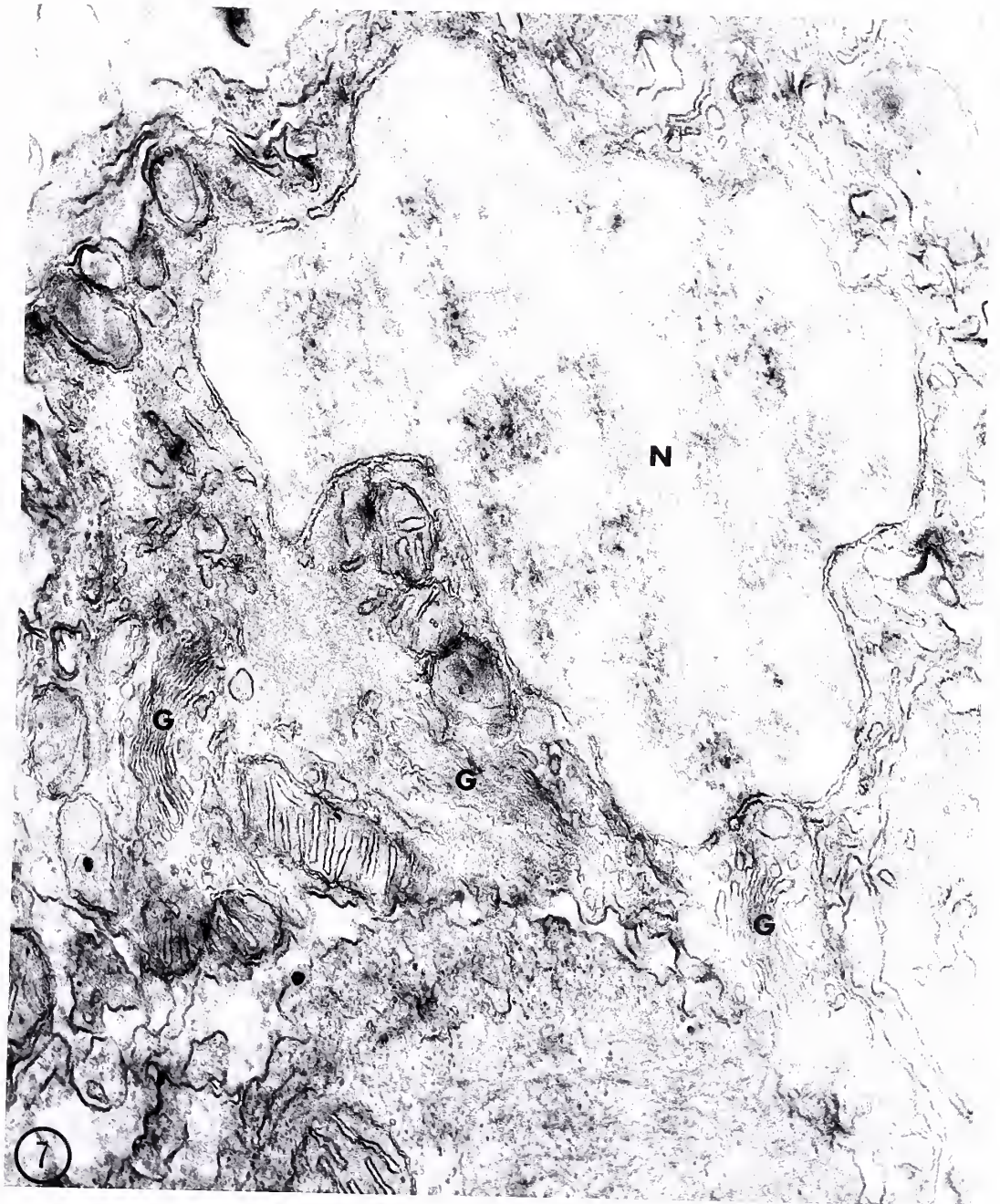


Fig. 8 Ganglion cell. Two complex Golgi apparati are situated near the nucleus. Several mitochondria are located at a greater distance from the nucleus. Note that two mitochondria are situated at the ends of the Golgi oriented parallel to the long axis of the cell. Many vesicles surround the second Golgi which is located between the nucleus and the process.

45,000X

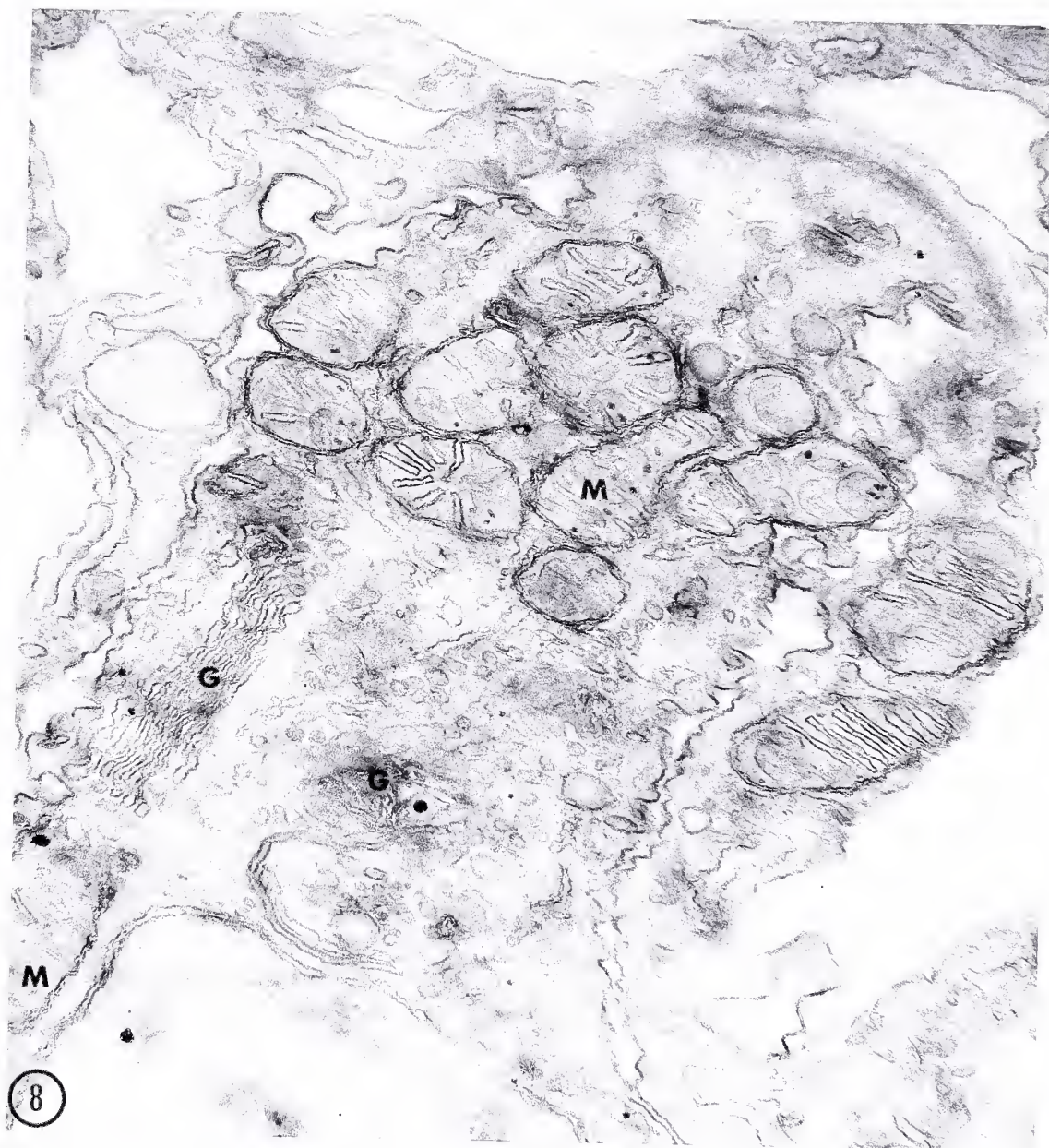


Fig. 9 Two ganglion cells. Both cells contain numerous ribosomes, microtubules, mitochondria, and vesicles. Note that the microtubules of one cell extend from the region of the nucleus out into the cell process. 45,000X

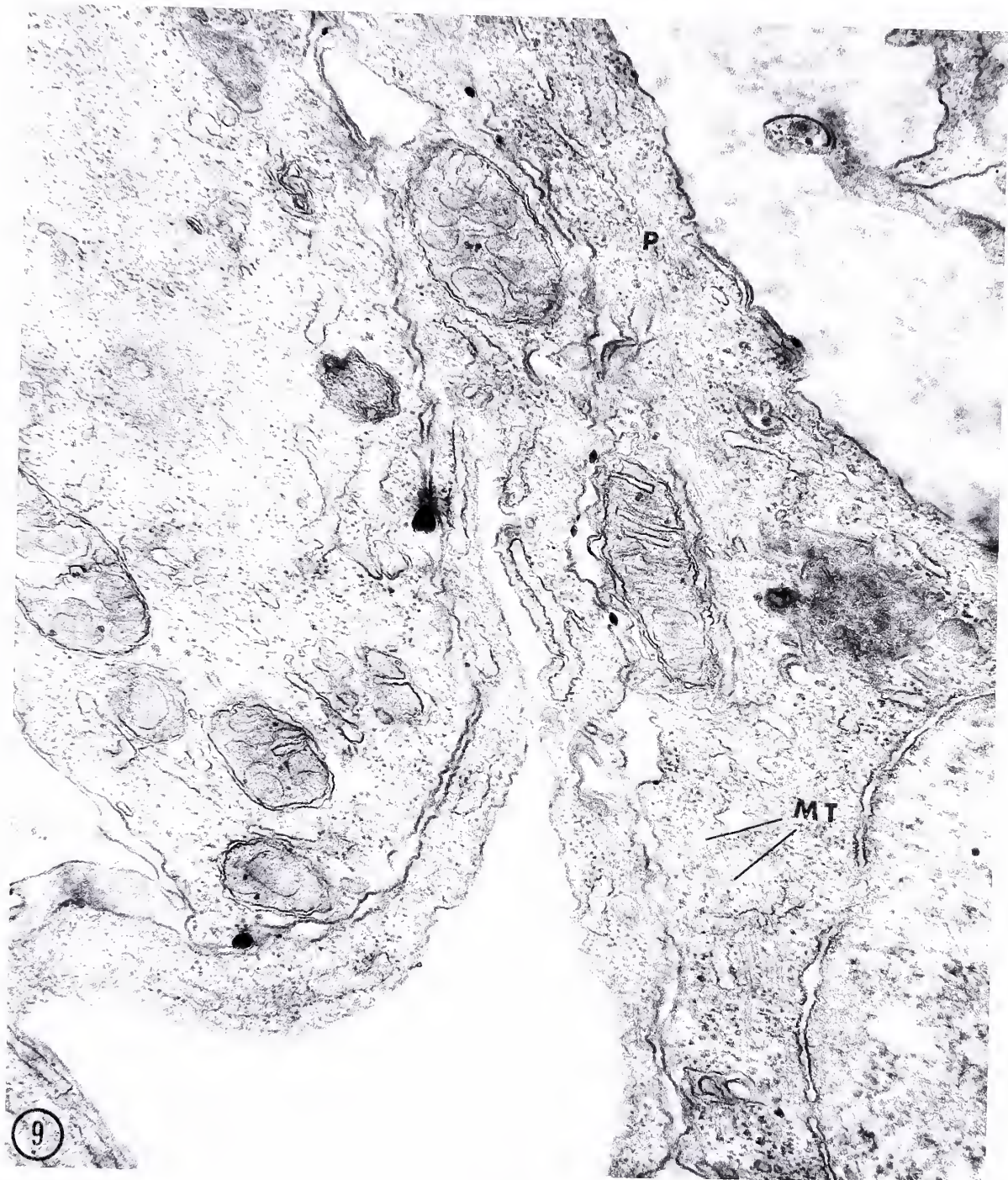


Fig. 10 Cross section through two nerve cell processes (neurites) adjacent to the base of a cnidoblast. The neurites contain vesicles and mitochondria. Dense granules are present in the intercellular spaces surrounding the neurites. Note the complex rough surfaced endoplasmic reticulum within the cnidoblast.
37,500X

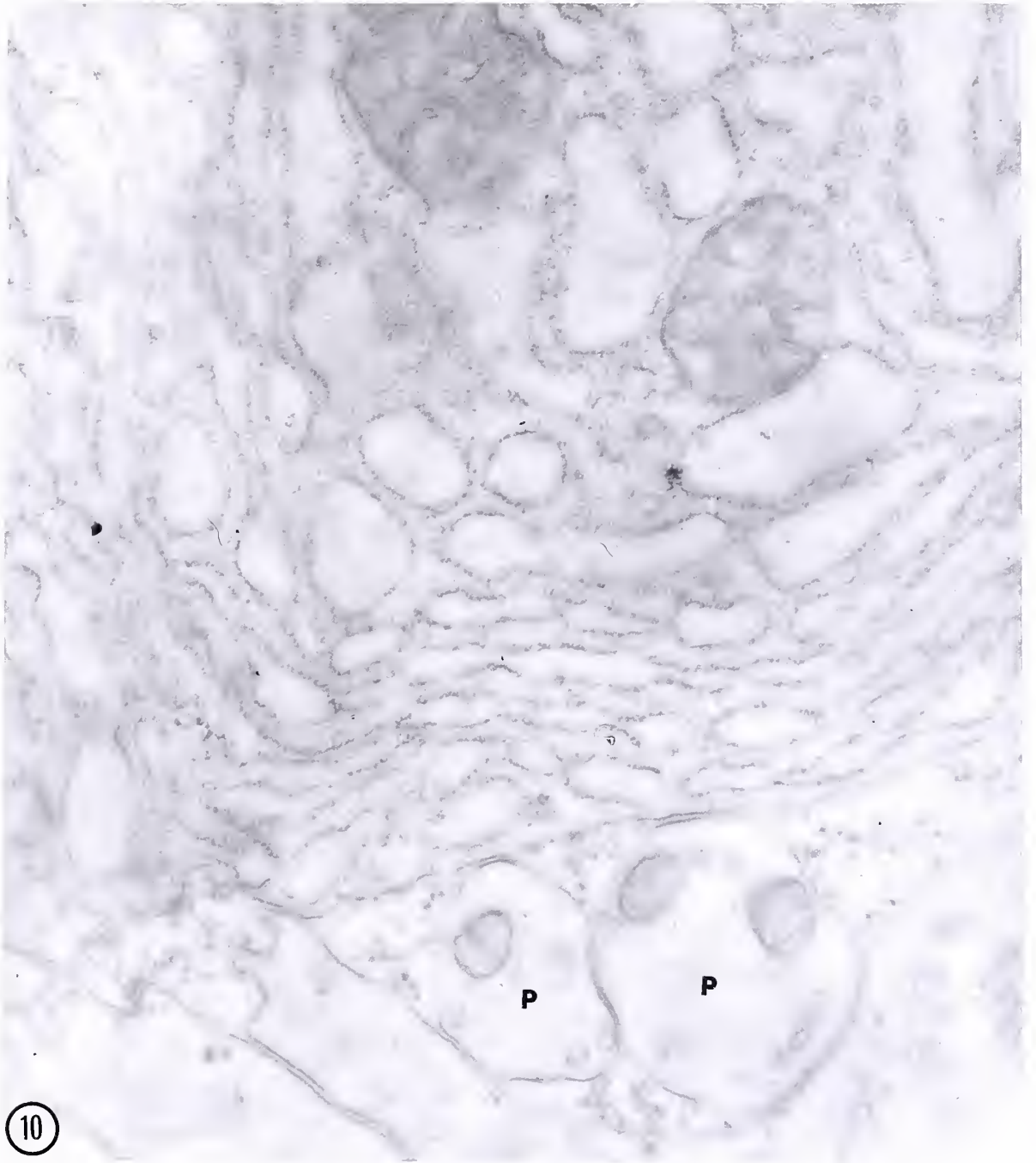
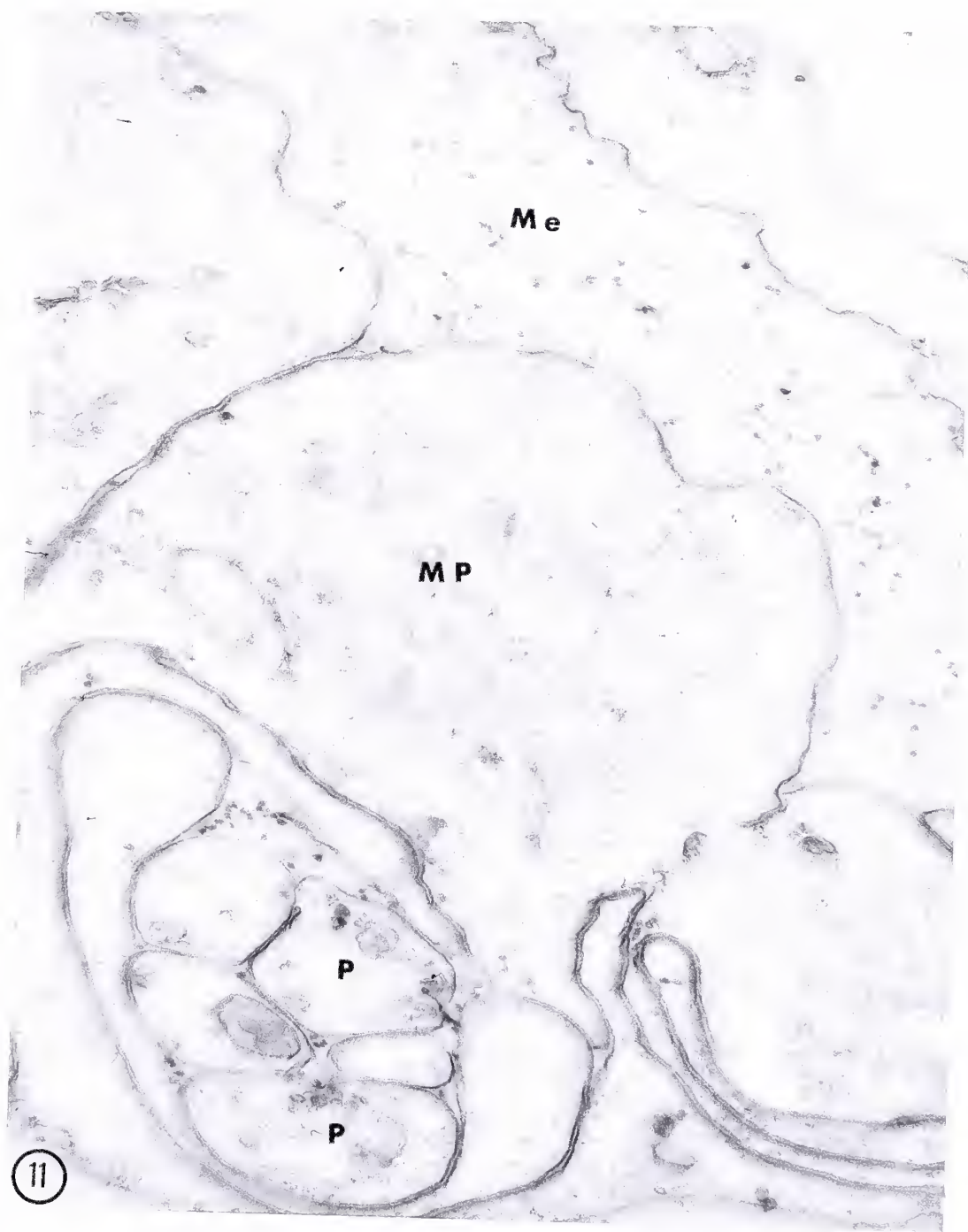


Fig. 11 Cross section through a bundle of neurites lying above the muscular processes of epitheliomuscular cells. Note that the neurites are surrounded by a process of another cell type. The neurites contain a few vesicles and dense granules. Dense granules are also present within the spaces between the neurites and in the mesoglea. 36,000X



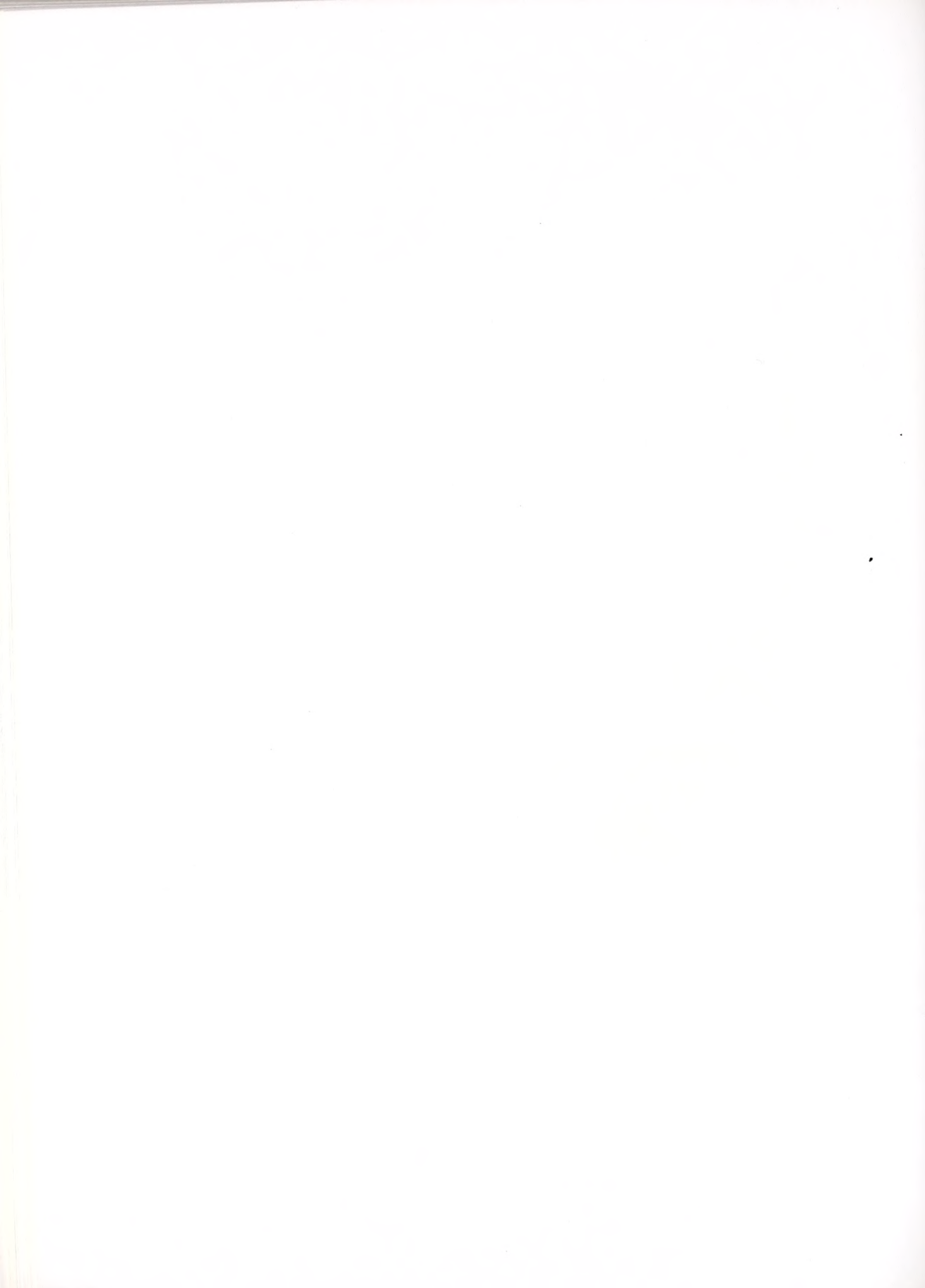


Fig. 12 Neurosecretory cell possessing a long process. Note the abundance of dense granules within the process. The process appears to terminate in three narrow constrictions which resemble intercellular spaces. 24,000X

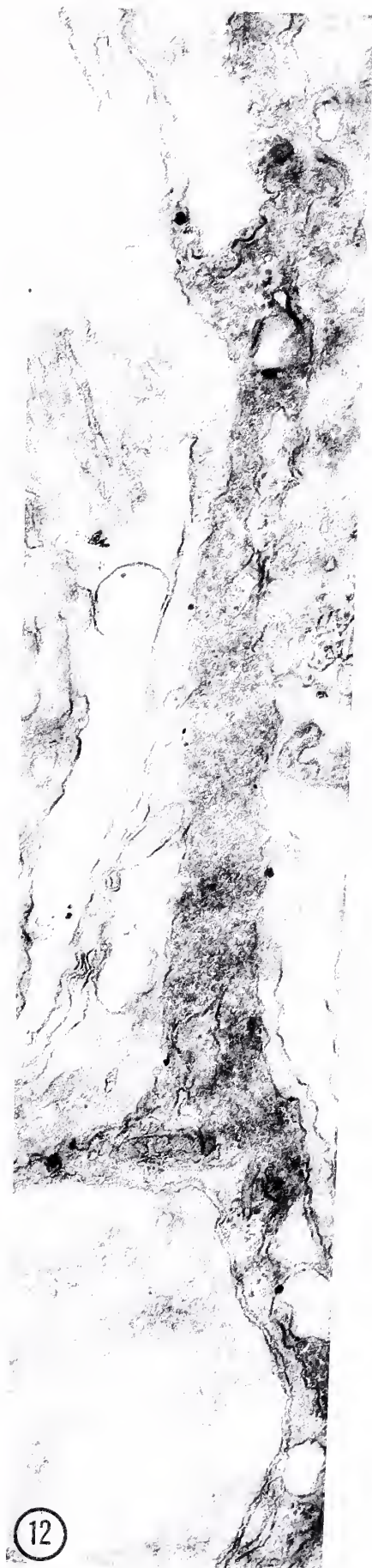


Fig. 13 High magnification of a neurosecretory cell. A Golgi apparatus is situated near the nucleus. A few dense granules are present within Golgi vesicles (arrows) but many more are present in a cell process at the top of the picture. The perikaryon also contains mitochondria, ribosomes, and endoplasmic reticulum. 67,000X

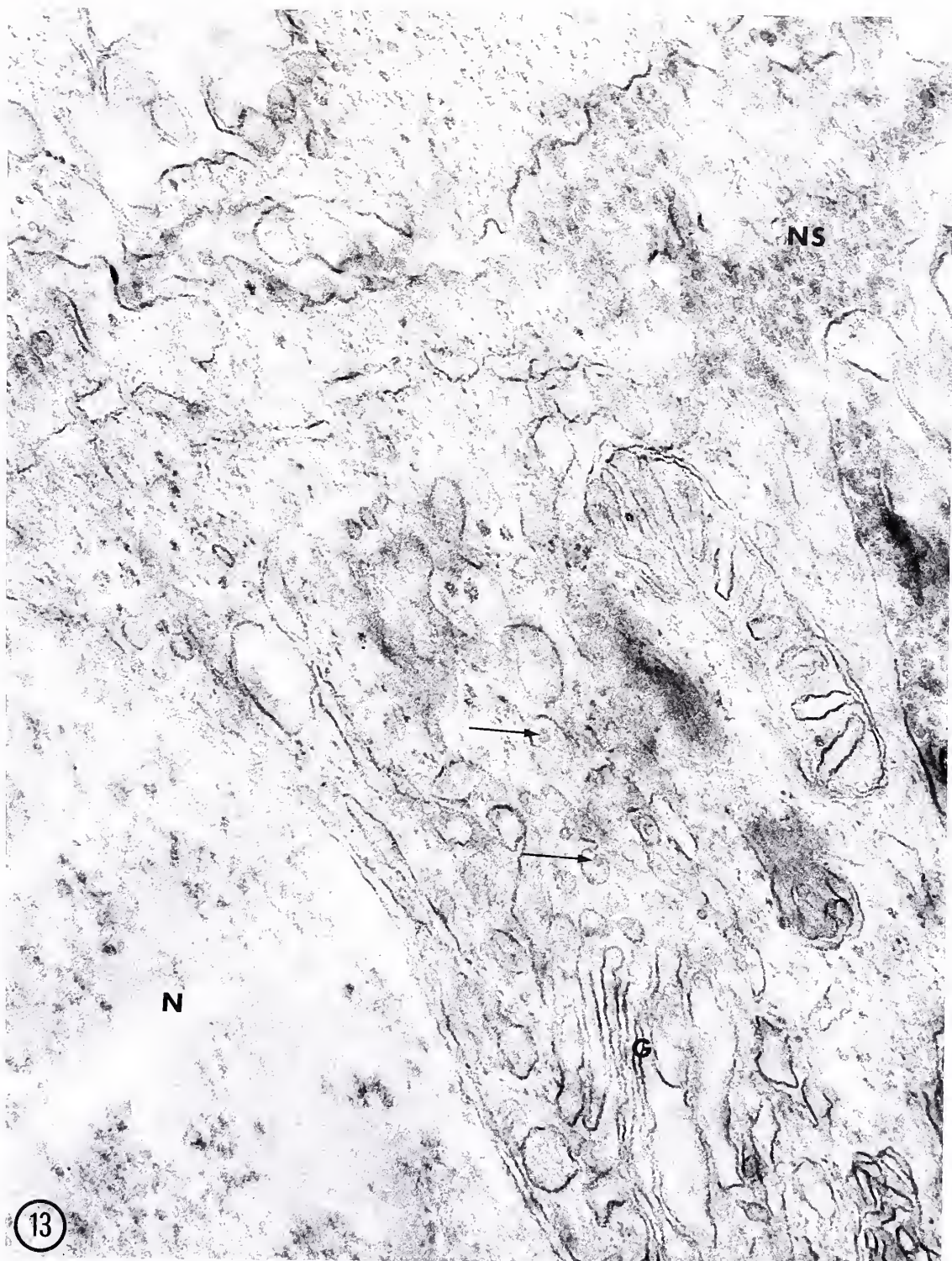


Fig. 14 Golgi apparatus and two mitochondria within a large process of a neurosecretory cell. Note that dense granules are present within the dilated ends of the parallel lamellae of the Golgi apparatus. Two membrane bound accumulations of granules are situated near the Golgi complex. A few dense granules are present in an intercellular space on the left.

104,000X

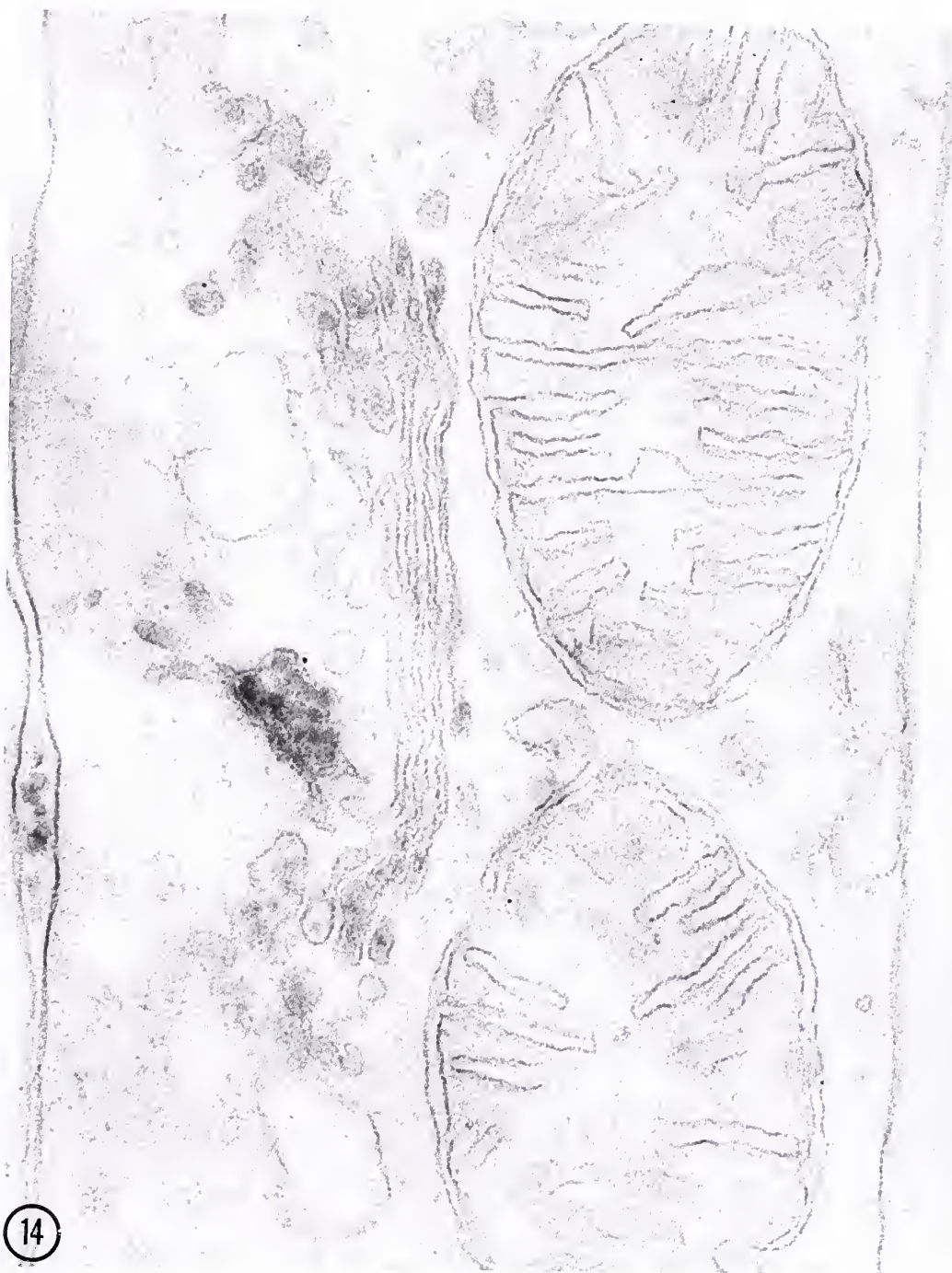
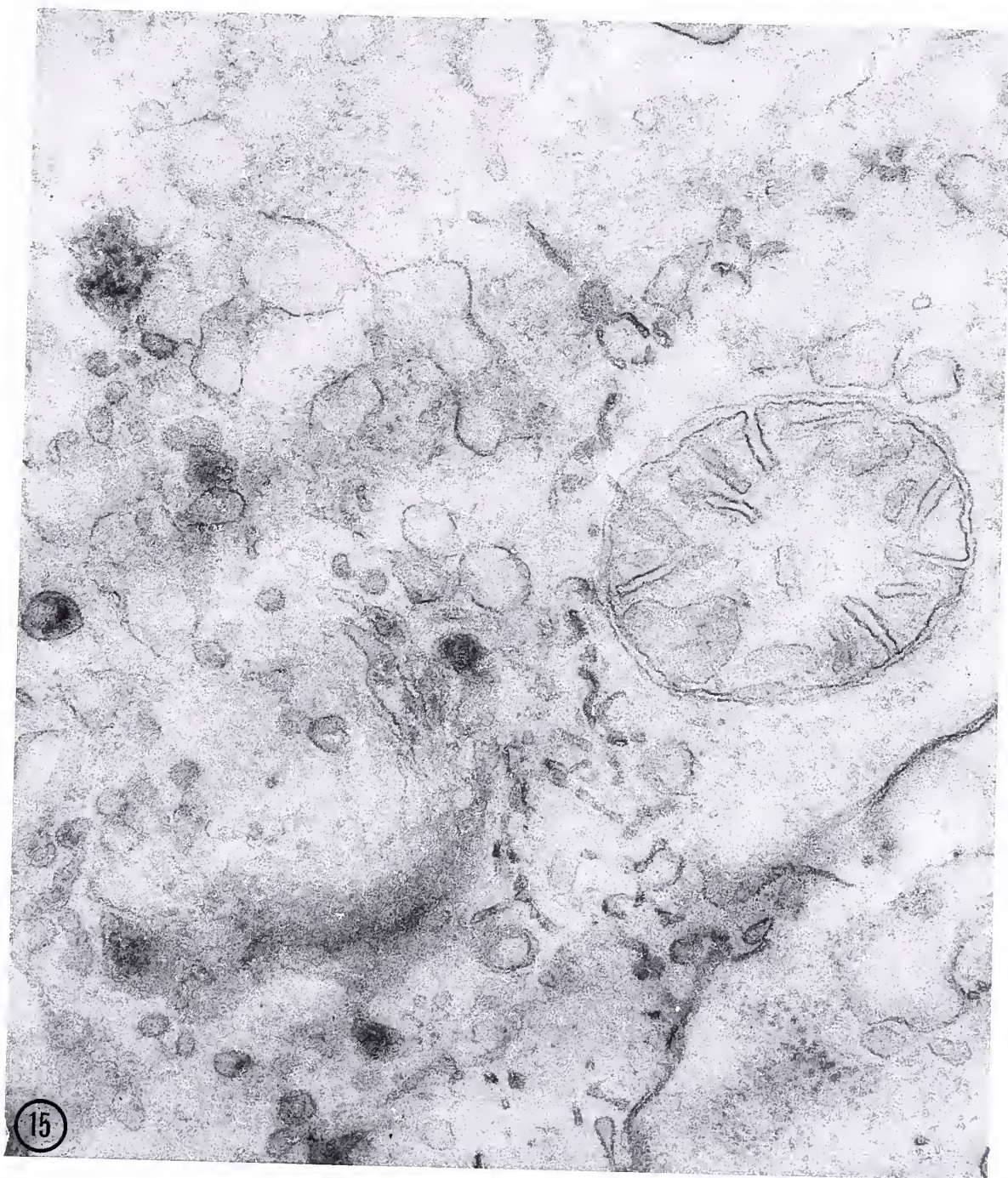


Fig. 15 Golgi apparatus located in the cytoplasm of a neurosecretory cell. Note that the ends of some of the Golgi lamellae are dilated and filled with dense granules. The surrounding cytoplasm contains small membrane-bound accumulations of granules. 92,000X






Fig. 16 Longitudinal section through a neurite. The neurite lies above the muscular process of an epitheliomuscular cell and contains membranous vesicles and dense granules. Similar granules are present within the mesoglea. 63,000X

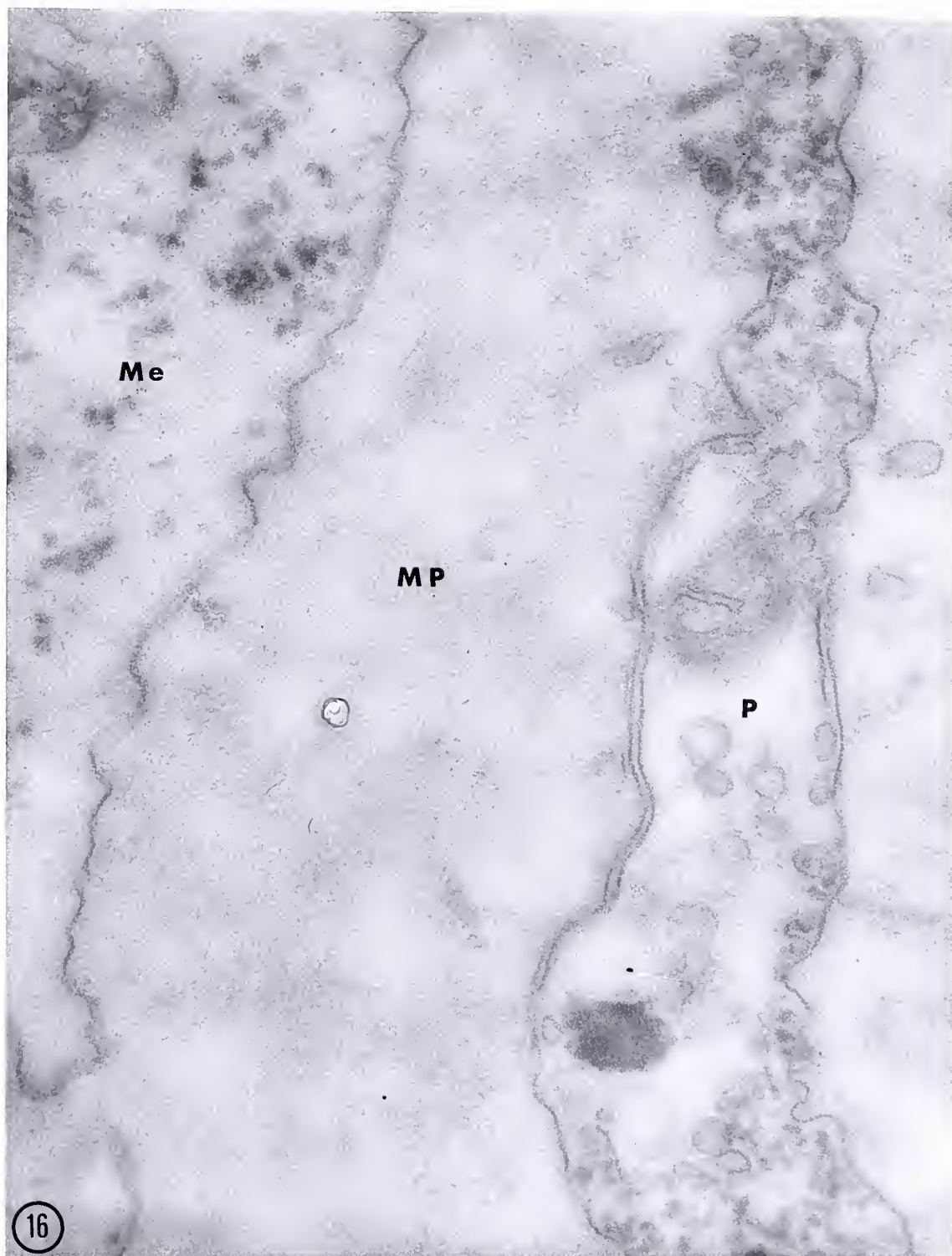


Fig. 17 Termination of the process of a neurosecretory cell. Note the abundance of dense granules. Three narrow constrictions arise from a bulbous enlargement. The narrow constrictions containing granules are indistinguishable from intercellular spaces. 65,000X

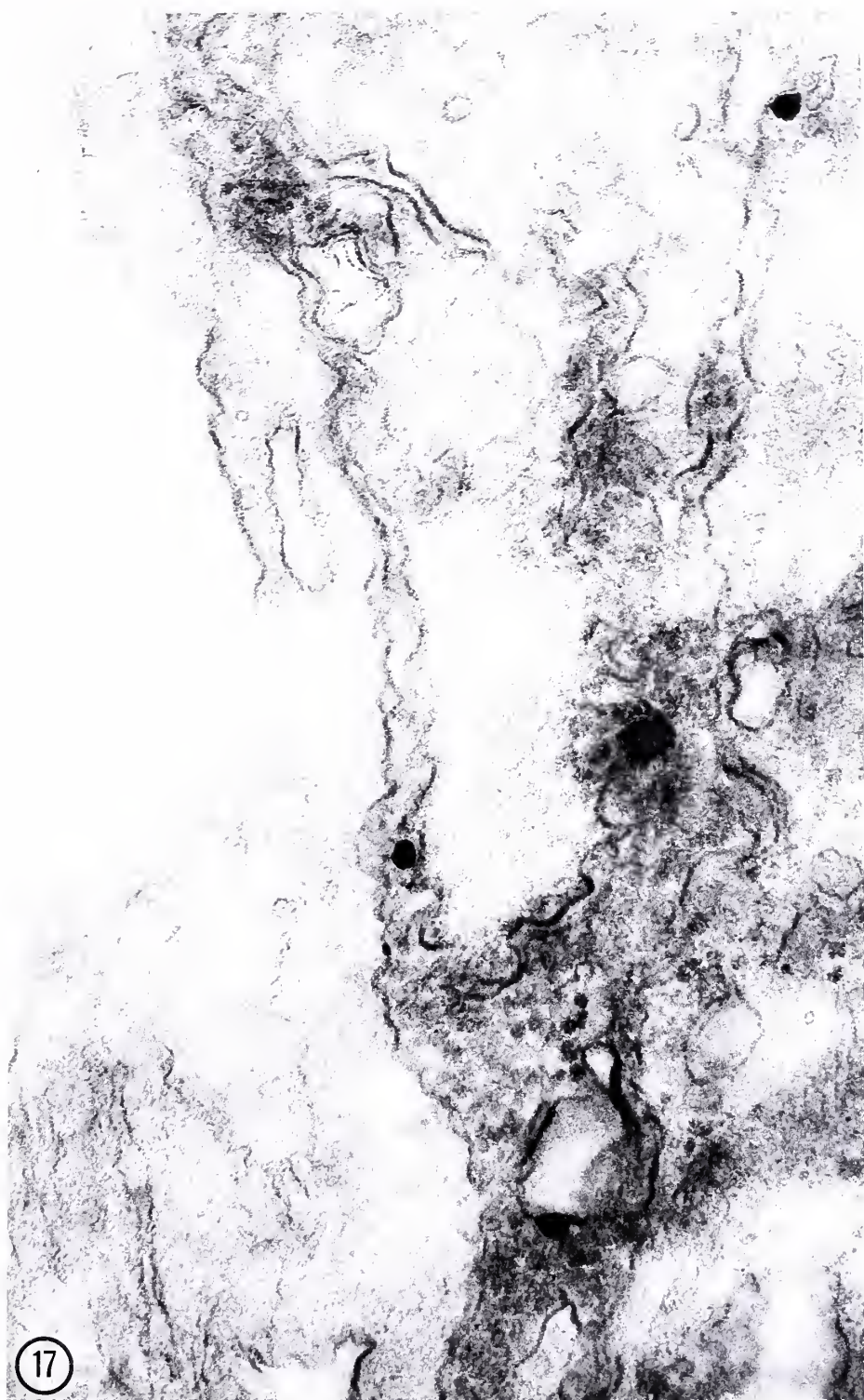


Fig. 18 Elongated sensory cell situated in the epidermis. Note the oval structure surrounded by a membranous sac at the apex of the cell. The cytoplasm contains mitochondria, a Golgi apparatus, and numerous vesicles. Dense granules are situated near the base of the cell. 19,000X

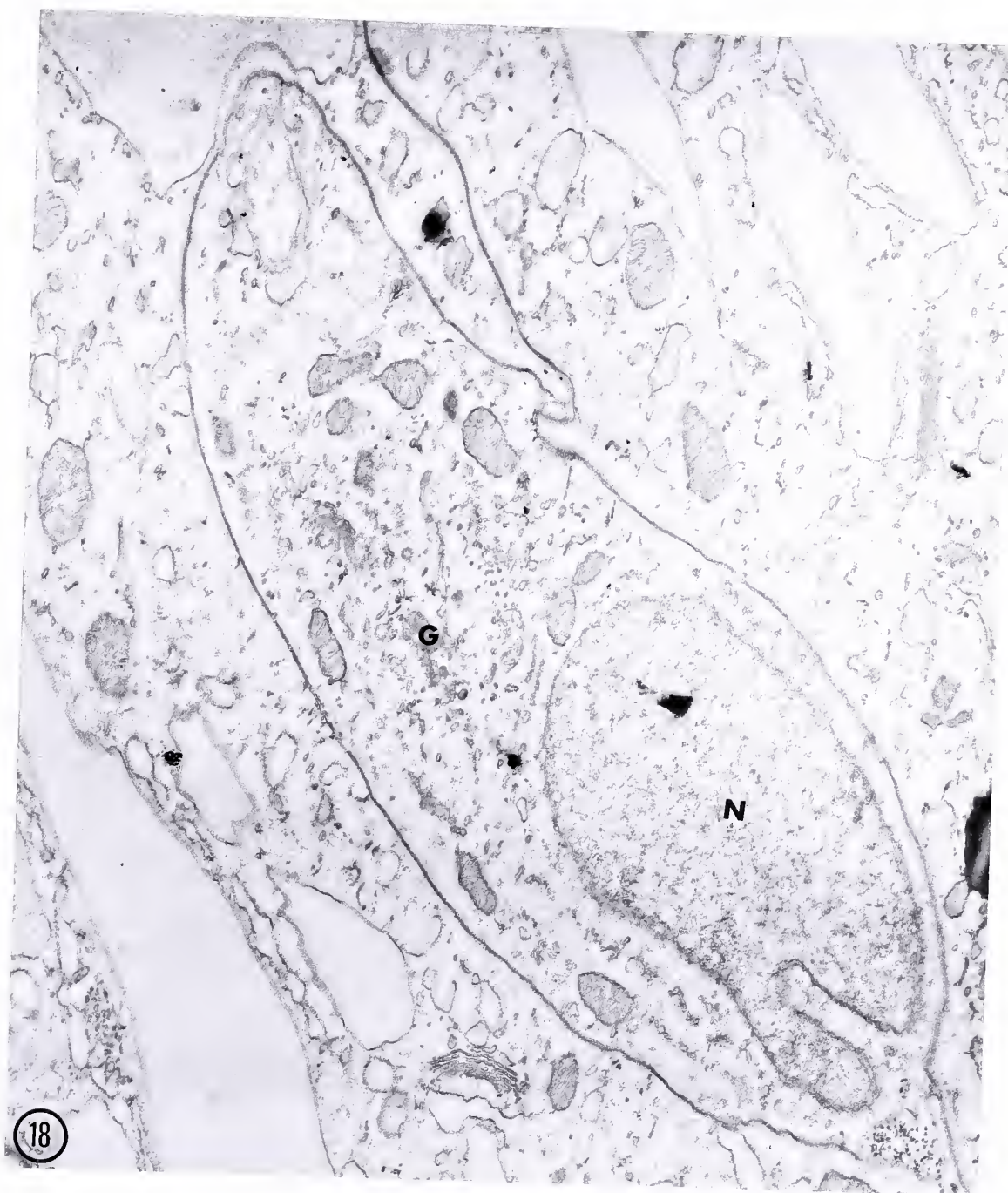
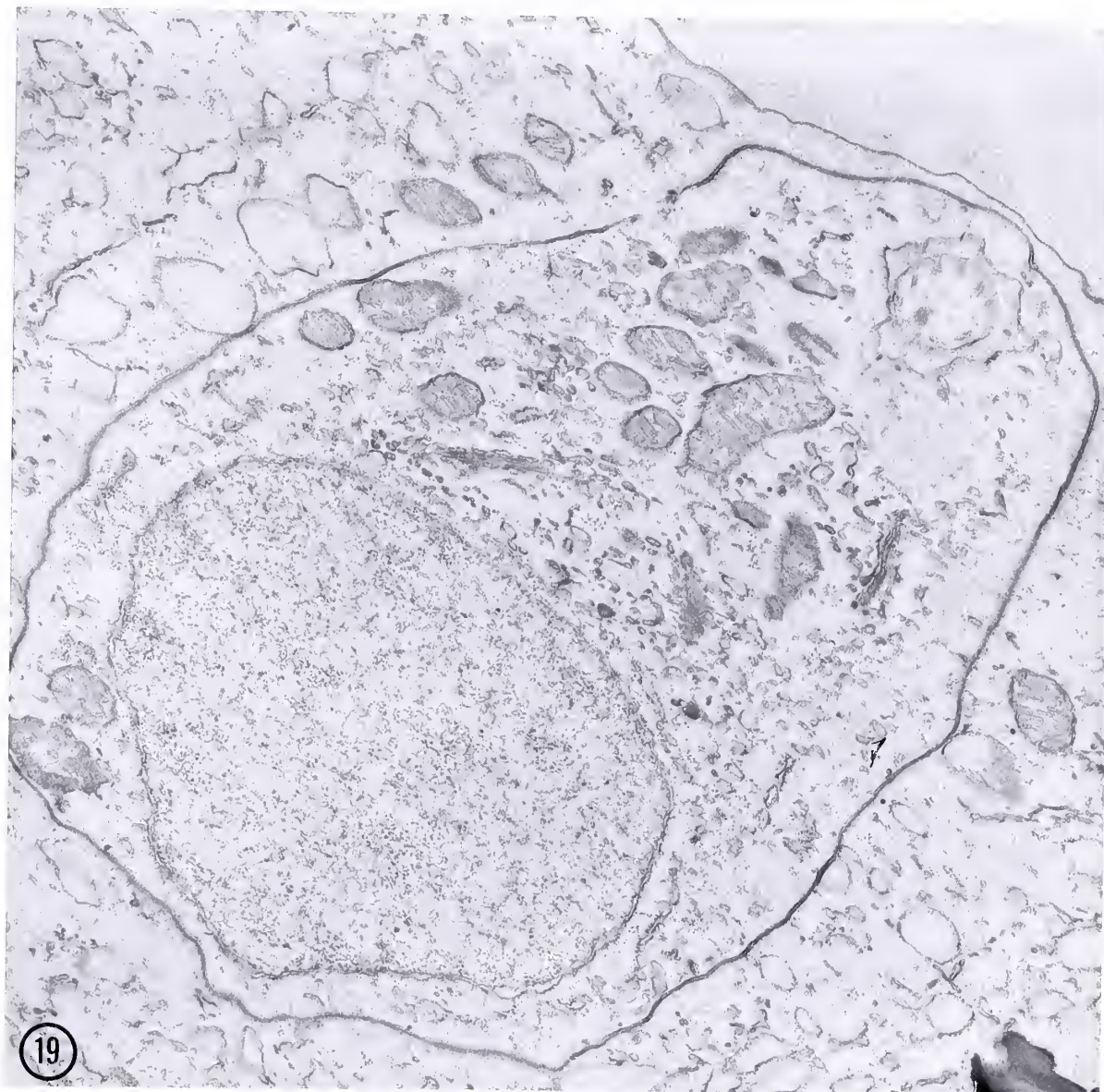


Fig. 19 Sensory cell embedded in an epitheliomuscular cell. The terminal structure contains rods and is surrounded by a membranous sac. A golgi apparatus is located above the nucleus. 19,000X



Chapter 10

The Relationship of Enzymatic Activity to Permeability

Investigation of the histochemical distribution of enzyme activity of hydra demonstrated the association of a variety of hydrolytic and oxidative enzyme activities with the cell surface of both epidermal and digestive cells (Chapter 3). Electron microscopic studies have revealed structural specializations of the epidermal and gastrodermal surfaces. The surface of the epidermis has two extraneous coats of material which presumably function to maintain the internal environment of the cell against the hypotonic external environment. The presence of vacuoles containing enzyme activity near the surface of epidermal cells suggested that selective uptake of some material by these cells was possible. The gastrodermal cells are specialized for the active uptake of material into the cell and they possessed numerous microvilli and pinocytotic or phagocytic invaginations. These surface specializations provided an opportunity for studying the relationship of enzyme activity to permeability since many enzymes were localized to the surface membranes. Therefore, to further investigate these processes, a study of the fine structural detail of the surface of the epidermis and gastrodermis was undertaken. This was followed by a study of ferritin uptake and the effects of enzyme inhibitors on the ferritin uptake by the cells of the epi-

dermis and gastrodermis.

Material and Methods

Hydra littoralis were maintained in culture as outlined in preceding chapters. Mature, non-budding hydra which had been fed 24 hours previously were selected for the present studies. The fine structural morphology of the apical surfaces of epidermal and gastrodermal cells was studied using glutaraldehyde and osmium tetroxide fixed material which had been embedded in Epon or Maraglas. These methods are described in Chapter 8. In order to study normal ferritin uptake up the epidermis, intact living hydra were placed in a solution of purified ferritin isolated from horse spleen (Pentex, Inc., Kankakee, Illinois) which had been diluted 1:1 with hydra culture water which contains versene (1:20,000) and CaCl_2 (1:4000). After 20 to 45 minutes in this solution, the hydra were fixed for electron microscopy. For study of the gastrodermis, the diluted ferritin solution was injected through the mouth into the digestive cavity with a short bevel #27 needle. Various inhibitors of some of the enzymes previously identified in the cell membrane (Chapter 3) were added in other experiments to the ferritin solution to study their effect on ferritin uptake by hydra cells. The following inhibitors were utilized: cysteine (10^{-3}M), sodium fluoride (10^{-3}M), phlorizin (10^{-3}M), N-ethyl maleimide (10^{-2}M), sodium taurocholate (10^{-2}M), and

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eserine (physostigmine) ($10^{-4}M$). All animals were fixed as described above. Thin sections were cut on a Porter-Blum microtome and examined with an RCA EMU 3F electron microscope.

Observations

The surface specializations of epidermal and gastroduermal cells are presented prior to a consideration of ferritin uptake. The most striking feature of the epidermal surface was the presence of a homogeneous non-opaque layer directly adherent to the plasma membrane. This in turn was covered by a finely granular and fibrillar layer of material on the external surface (Figs. 1 - 5). This layer was approximately 0.6μ thick and covered the entire epidermal surface except the base where a thick mucoid layer occurred. The plasma membrane was a typical unit membrane composed of two dense layers on both sides of a thicker, less dense layer. The apical membrane was relatively smooth in contour bearing few invaginations and no microvilli except in the region of the base where the latter were numerous and extended into the mucoid layer.

Numerous small vesicles were present in the apex of the cytoplasm immediately below the plasma membrane (Fig. 1). Larger vacuoles and membrane-bound intracellular spaces were present deeper in the cytoplasm. Mucous granules, membrane-bound accumulations of finely granular material, were also present below the plasma membrane

From the 1st of January to the 31st of December
the total number of cases of smallpox was 100
and the total number of deaths was 10.

The following table shows the number of cases of smallpox
and the number of deaths from smallpox in each of the
years from 1880 to 1890. The number of cases of smallpox
in each year is given in the first column, and the number of
deaths from smallpox in each year is given in the second column.
The total number of cases of smallpox from 1880 to 1890 is 1000,
and the total number of deaths from smallpox from 1880 to 1890 is 100.
The following table shows the number of cases of smallpox
and the number of deaths from smallpox in each of the
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in each year is given in the first column, and the number of
deaths from smallpox in each year is given in the second column.
The total number of cases of smallpox from 1880 to 1890 is 1000,
and the total number of deaths from smallpox from 1880 to 1890 is 100.
The following table shows the number of cases of smallpox
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The total number of cases of smallpox from 1880 to 1890 is 1000,
and the total number of deaths from smallpox from 1880 to 1890 is 100.

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years from 1880 to 1890. The number of cases of smallpox
in each year is given in the first column, and the number of
deaths from smallpox in each year is given in the second column.
The total number of cases of smallpox from 1880 to 1890 is 1000,
and the total number of deaths from smallpox from 1880 to 1890 is 100.
The following table shows the number of cases of smallpox
and the number of deaths from smallpox in each of the
years from 1880 to 1890. The number of cases of smallpox
in each year is given in the first column, and the number of
deaths from smallpox in each year is given in the second column.
The total number of cases of smallpox from 1880 to 1890 is 1000,
and the total number of deaths from smallpox from 1880 to 1890 is 100.

(Figs. 1 - 3) and extremely numerous in the cells of the base where they filled the entire apical cytoplasm. Mitochondria and a scattering of ribosomes were also present in the apical cytoplasm.

The junctions of adjacent epitheliomuscular cells were characterized by desmosomal-like structures near the surface (Figs. 3 - 5). These structures consisted of a thickening of adjacent cell membranes and transverse bars extending across the space between the two membranes. A short distance below the desmosomes intracellular spaces were formed by a separation of the adjacent membranes (Figs. 3 and 6).

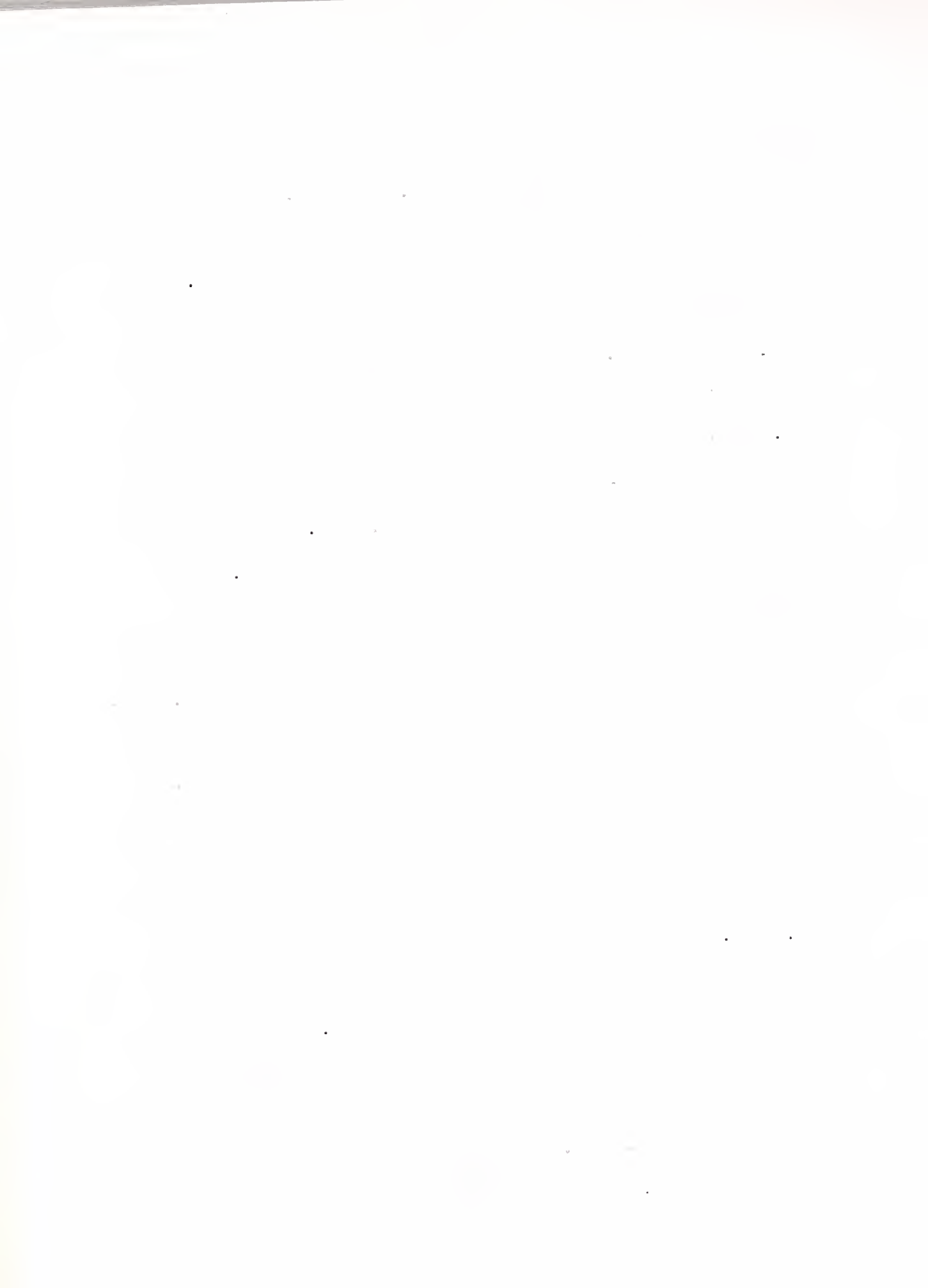
In marked contrast to the epidermis, cells of the gastrodermis possessed numerous microvilli of varying lengths extending into the digestive cavity (Figs. 7 - 12). A loose feltwork of fibrillar material covered the plasma membranes of the gastrodermal cells. There was no distinguishable morphological difference between the plasma membrane of these cells which extended over the microvilli and the plasma membranes of the epidermal cells. Pinocytotic invaginations were often present at the bases of the microvilli. Small, membrane-bound cytoplasmic channels were present near the cell surface and frequently communicated between the cell surface and larger vacuoles located deeper in the cytoplasm.

The apex of the cells contained numerous dense

granules, presumably glycogen, free in the cytoplasm and within membranous envelopes (Figs. 7 - 10). Mitochondria and large vacuoles containing particulate matter were also present in the apical cytoplasm of digestive cells. Pairs of flagella extended from the apex of gastrodermal cells (Figs. 10 and 11). In cross section, the flagella contained two central rods surrounded by nine outer pairs (Fig. 11). Each cell possessed from one to at least four pairs of flagella. Intercellular attachments were identical to those of epidermal cells (Fig. 12). Intercellular spaces were present below the apical desmosomes.

Ferritin uptake - Epidermis

The epidermal cells were impermeable to ferritin, none being present in the cell after 20 minutes (Fig. 13). A few ferritin particles, however, were adherent to the external fibrillar layer of the extraneous coat (Fig. 13). With longer exposure to the ferritin solution, long slender surface processes extended and enveloped a portion of the external fibrillar layer and its attached particles (Fig. 14). By this process, large membrane-bound cytoplasmic vacuoles containing ferritin were formed, but these were not incorporated into the cell. Instead, the projection pinched off at the base and formed extra-cellular vacuoles of ferritin enclosed in an ejected piece of cytoplasm (Fig. 15). In some cases, these bodies were connected to the epidermis proper by a narrow cytoplasmic



bridge which had not yet pinched off.

Gastrodermis

The digestive cells rapidly took up large quantities of ferritin in contrast to the epidermal cells (Figs. 16 - 19). Ferritin first appeared attached to the external fibrillar feltwork coating the cell surfaces and numerous microvilli (Figs. 16 and 17). Pinocytotic vesicles and invaginations at the bases of the microvilli were usually filled with ferritin (Figs. 16 and 17). A few cytoplasmic channels also contained ferritin. These channels and invaginations pinched off from their surface connections so that by 20 minutes large agminations of ferritin with membrane-bound vesicles were present (Fig. 16). After 45 minutes, very little ferritin was present on the cell surface or within pinocytotic invaginations (Figs. 18 and 19). Instead, the deeper portions of the cytoplasm contained many small cytoplasmic channels and cytoplasmic vesicles containing ferritin and both of these were frequently connected to large dense accumulations of ferritin within membrane-bound vacuoles (Figs. 18 and 19). These vacuoles appeared to concentrate the ferritin particles since over the time space of the experiments more particles appeared to be contained in the vacuolar spaces.

Effect of enzyme inhibitors on ferritin uptake

None of these agents employed had any effect,

TABLE 1

Effect of enzyme inhibitors on the uptake of ferritin by digestive cells of hydra. Only those enzymes known to be present in the cell membranes of hydra are listed.

Inhibitor	Enzyme Inhibited At Surface	Effect on Ferritin Uptake
Cysteine (10^{-3}M)	Alkaline phosphatase	No uptake
N-ethyl maleimide (10^{-2}M)	ATPase DFNH diaphorase TFNH diaphorase	No uptake
Fluoride (10^{-3}M)	ATPase Acid phosphatase Glucose-6-phosphatase 5'-nucleotidase Non-specific esterase	Slight uptake
Phlorizin (10^{-3}M)	ATPase	Slight uptake
Physostigmine (10^{-4}M)	Acetylcholinesterase	Normal uptake
Taurocholate (10^{-2}M)	Non-specific esterase	Increased uptake



either stimulatory or inhibitory, on the relative impermeability of the epidermis to ferritin. The observations on the effect of enzyme inhibitors on known (Chapter 3) enzymes at the surface of digestive cells are summarized in Table 1 and are presented below.

Cysteine ($10^{-3}M$) which inhibits alkaline phosphatase but not nucleotide phosphatase activity had marked effects on gastrodermal uptake almost completely inhibiting entrance of ferritin into the cells (Fig. 20). Some ferritin was attached to the cell surface and rarely small amounts were present within cytoplasmic channels. Vesicles and vacuoles containing ferritin were absent for the most part.

The effects of N-ethyl maleimide ($10^{-2}M$) which inhibits sulfhydryl dependent enzymes were more marked than those of cysteine. Even less ferritin entered the cell in the presence of this inhibitor.

Sodium fluoride ($10^{-3}M$) was only a moderately effective inhibitor of ferritin uptake (Fig. 21). A few large accumulations of ferritin were present although not nearly as numerous as in the uninhibited cells. Ferritin was attached to cell surfaces and present within a few vesicles and cytoplasmic channels.

Phlorizin ($10^{-3}M$) which inhibits some nucleotide phosphatases inhibited ferritin uptake to a degree equivalent to that of sodium fluoride (Fig. 22). Ferritin

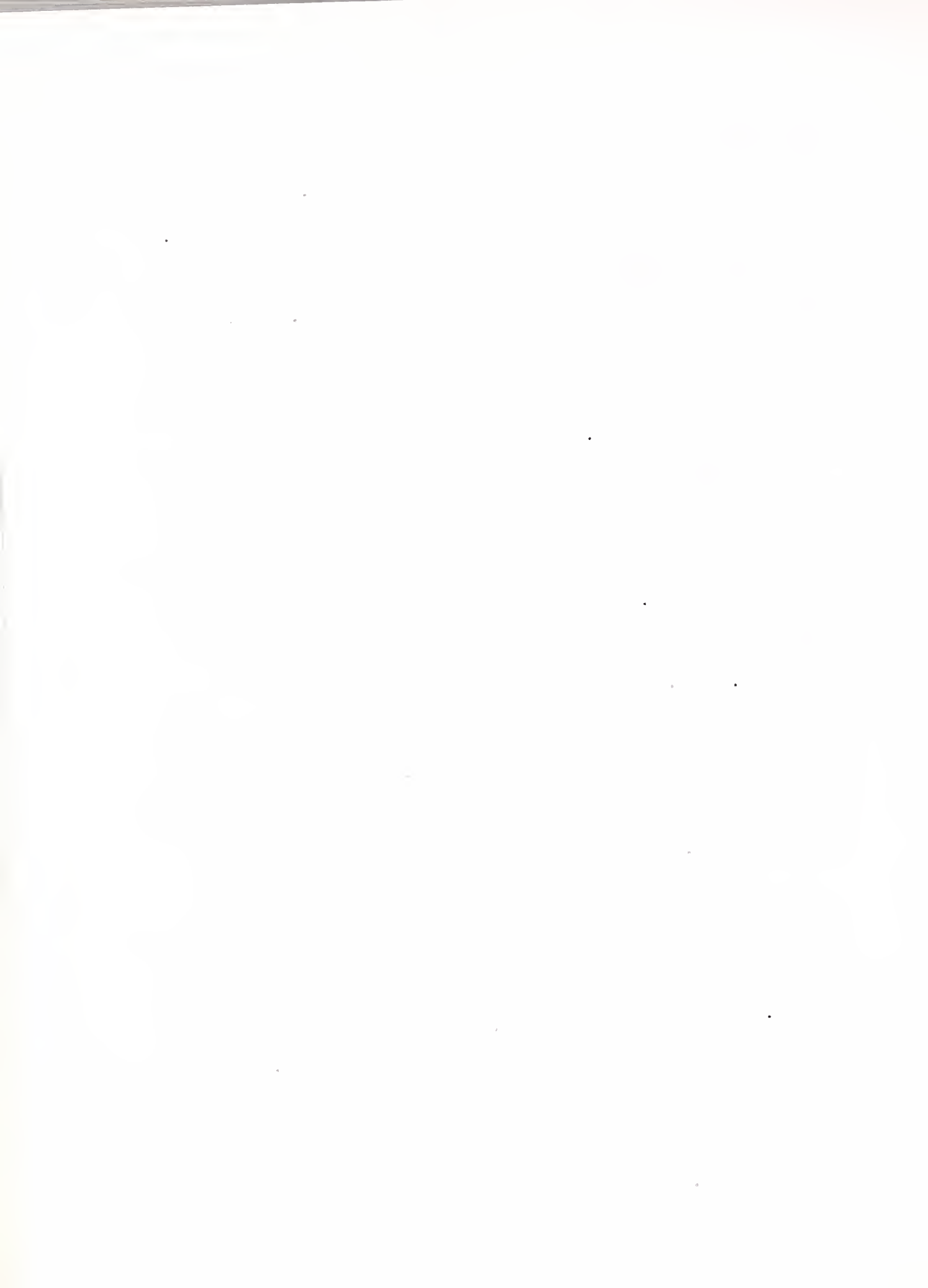
was attached to cell surfaces and present within pinocytotic vesicles and cytoplasmic channels. However, massive accumulations were rare and widely scattered.

Physostigmine (eserine) ($10^{-4}M$) which inhibits AChE had no effect on ferritin uptake (Fig. 23). Ferritin proceeded normally from pinocytotic invaginations, through cytoplasmic channels, and into large membrane-bound accumulations.

Sodium taurocholate ($10^{-2}M$) which inhibits non-specific esterases but stimulates lipase activity, contrary to the other agents employed, resulted in increased ferritin uptake. After 20 minutes, large membrane-bound spaces close to the cell membrane were filled with ferritin (Fig. 24). These accumulations were as large and occasionally larger than those seen in the normal hydra exposed to ferritin for 45 minutes. Ferritin apparently entered these spaces in a normal manner by way of vesicles and channels.

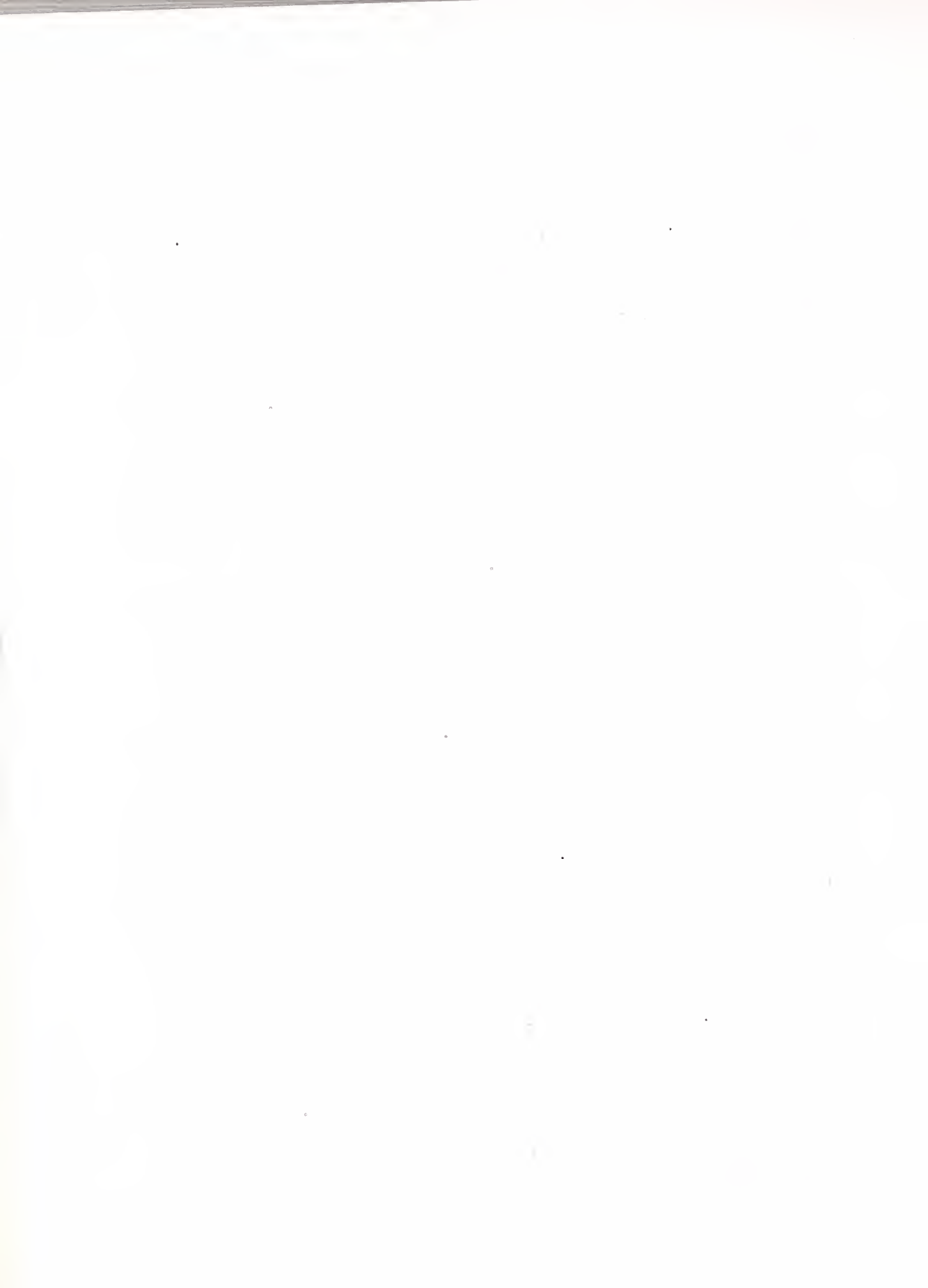
Discussion

The responses of the epidermis and gastrodermis to ferritin illustrate some of the functions of these cell layers. The epidermal cells appeared primarily to exclude ferritin from the cytoplasm of the cell. Presumably, this function rests with the external protective coating, since ferritin became embedded in this matrix but did not pass through. The occasional outgrowth of cytoplasmic



processes around ferritin particles represented the first stage in what might have been the uptake of ferritin by phagocytosis. However, the process went no further. Instead, the cytoplasmic processes surrounding the ferritin were pinched off. This process may represent an attempt by the cell to rid itself of particulate material which has been trapped in its outer matrix coating. Therefore, it appears that a primary function of the epidermal surface is to provide an external barrier to its environment and this includes the external protective layer which may act as a mechanical barrier.

While the epidermis appeared specialized to exclude particles, the digestive cells were specialized for the rapid incorporation of large amounts of material introduced into the digestive cavity. Ferritin particles were first trapped and held by the external feltwork coating at the apical surface of the cell membranes prior to their entrance into the cell. In this regard, Brandt and Pappas ('60) have shown that the initial step in the pinocytosis of ferritin and thorium dioxide by amoeba is attachment of these substances to the hair-like extensions of the plasmalemma. In addition, Gauthier ('63) observed in hydra the trapping of small lipid droplets by this layer prior to their incorporation by pinocytosis. Following entrapment at the surface, pinocytotic or phagocytic invaginations at the bases of the microvilli provided a



route of entry of ferritin into the cell. These invaginations presumably pinched off to form ferritin containing, membrane-bound vesicles within the cytoplasm or in some cases they enlarged to form cytoplasmic channels extending between the cell surface and intracellular vacuoles.

Similar mechanisms may operate during normal feeding. Large crustaceans brought into the digestive cavity during feeding are partially broken down by enzymes released into the cavity by gland cells. Pinocytotic and phagocytic invaginations and channels could provide entry for the partially digested material. Both vesicles and channels are incorporated into large vacuoles where intracellular digestion presumably takes place.

The presence of enzyme activities in or near the cell surfaces suggested that these may play a role in the process just described. This suggestion is strengthened by the finding that enzyme inhibition resulted in the inhibition of ferritin uptake. Ferritin uptake by digestive cells was inhibited in varying degrees by cysteine, N-ethyl maleimide, sodium fluoride, and phlorizin. These substances have been shown to inhibit the activities of alkaline phosphatase, TPNH and DPNH diaphorases, acid phosphatase, glucose-6-phosphatase, non-specific esterase, 5-nucleotidase, and adenosinetriphosphatase in the hydra (Chapter 3). Although some or all of these enzyme activities may play a role in the entrance of substances into

house of 1000 or 1200 ft. high, and 1000 ft. long.

There are many other things to see here.

The first thing I saw when I entered the house

was a large hall with a high ceiling.

The walls were covered with tapestries.

The floor was made of polished stone.

The ceiling was painted to look like a sky.

The furniture was made of dark wood.

The food was very good and plentiful.

The people were very friendly and hospitable.

The house was very comfortable and cozy.

The garden was very beautiful and well-kept.

The house was very large and spacious.

The house was very clean and tidy.

The house was very well-ventilated.

The house was very well-lit.

The house was very well-kept.

The house was very well-maintained.

The house was very well-looked after.

The house was very well-attended.

The house was very well-served.

The house was very well-cared for.

The house was very well-looked after.

The house was very well-maintained.

The house was very well-looked after.

The house was very well-maintained.

the digestive cell of hydra, attention should be directed especially to various phosphatases, especially nucleotide triphosphatases (ATPase). It first should be noted that the inhibitors did not affect in any way the protective action of the epithelial cells further strengthening the argument that this layer serves as an impermeable barrier which protects the animal from its aqueous environment. Taurocholate resulted in increased uptake and although this substance inhibits esterase and stimulates lipase activity, its effect is presumably due to its action as a surfactant.

Concerning the relationship of a phosphatase to transport phenomena, Danielli ('52) has suggested that the phosphatases located in the brush border of proximal kidney tubules, the striated border of intestinal mucosa, and in capillaries might be involved in active transport of substances across the cell membrane. However, Rothstein and his co-workers ('53) found that molybdate completely inhibited the phosphatases in the brush border of intestinal epithelium but did not affect glucose or phosphate uptake. This suggested that the enzymes located in the cell surface split phosphorylated sugars into orthophosphate and organic residue which were then absorbed by a separate mechanism. Miller and Crane ('61) felt that this mechanism constituted an efficient coupling of digestive hydrolysis and active absorption.

ATPase activity has been demonstrated in the cell membranes of erythrocytes (Glynn, '57; Post et al., '60), liver cells (Essner et al., '58), kidney tubule cells (Spater et al., '58), and pinocytotic vesicles of blood capillaries (Marchesi and Barrnett, '63). ATPase activity is present in cell membrane fractions as assayed biochemically (Acs et al., '55; Emmelot and Bos, '62; Cummins and Hyden, '62). Most of these investigators have suggested that the ATP/ATPase system functions to provide energy for transport.

On the other hand, the enzyme activity similar to the one demonstrated by Skou ('57, '62), a Na/K activated ATPase, cannot be ruled out. Even more appealing from a morphological point of view would be an ATPase linked enzyme system as first described by Hokin and Hokin ('60, '61) concerned with membrane synthesis. According to the present morphological evidence, the ferritin was taken up by pinocytosis and/or phagocytosis; that is, the ferritin was always enclosed in a membrane-bound envelope. It is suggested that if any adjustment is required of the gastrointestinal cells during ferritin uptake it is the synthesis of surface membrane via phosphatidic acid.

Some of the inhibitors used will also affect some enzymes of the respiratory chain and these, too, may be indirectly implicated in the uptake of ferritin by hydra cells. Presumably, if the transport of ferritin is also

linked to oxidative phosphorylation or to oxidative metabolism involving the electron transfer system, inhibition of these systems would also inhibit uptake by at least reducing the ATP available for energy reaction. After all, the apex of a gastrodermal cell is not as static as that revealed by electron micrographs or other morphological evidence. During the active uptake of material, these cells must have actively moving processes (both morphologically and histochemically) to accomodate the dynamic events described.

Summary

1. A study of the fine structural specializations of the surface of the epidermis and gastrodermis, uptake of ferritin, and the effects of enzyme inhibitors on the ferritin uptake by the cells of the epidermis and gastrodermis are reported in this chapter.
2. The most striking feature of the epidermal surface was the presence of a homogeneous, non-opaque layer directly adherent to the plasma membrane which in turn was covered by a finely granular and fibrillar layer of material on the external surface.
3. The gastrodermis possessed numerous microvilli extending into the gastrovascular cavity. A loose feltwork of fibrillar material covered the plasma membranes of the gastrodermal cells. Pinocytotic invaginations were often present at the bases of the microvilli. Small membrane-

bound cytoplasmic channels frequently communicated between the cell surface and larger vacuoles located deeper in the cytoplasm.

4. The digestive cells rapidly took up large quantities of ferritin in contrast to the epidermal cells which were impermeable to ferritin. Ferritin first appeared attached to the external fibrillar feltwork coating the cell surfaces. Pinocytotic invaginations were usually filled with ferritin. Later, many small cytoplasmic channels contained ferritin and these were frequently connected to large dense accumulations of ferritin within membrane-bound vacuoles.

5. Cysteine, which inhibits alkaline phosphatase, and N-ethyl maleimide, an inhibitor of ATPase and DPNH and TPNH diaphorases, completely inhibited ferritin uptake by digestive cells. Ferritin uptake was decreased in the presence of fluoride and phlorizin which inhibit ATPase. Physostigmine had no effect while taurocholate resulted in increased uptake.

6. These results suggest that enzymes present in the cell surfaces play a role in the uptake of ferritin by these cells. Although some or all of these enzymes (Chapter 3) may play a role in the entrance of substances into the digestive cells of hydra, particular attention should be directed to ATPase. It is suggested that if any adjustment is required of the digestive cells during ferritin uptake it is the synthesis of surface membrane via phos-

phatidic acid, an ATPase linked enzyme system. In addition, if the transport of ferritin is also linked to oxidative phosphorylation or to oxidative metabolism involving the electron transfer system, inhibition of these systems would also inhibit uptake by reducing the ATP available for energy reaction.

Abbreviations for Electron Micrographs

C	capsule	Mu	mucous granule
CC	central core	MV	microvillus
EFL	external fibrillar layer	N	nucleus
EPL	external protective layer	NC	nerve cell
ER	endoplasmic reticulum	Ne	nematocyst
F	lipid droplet	Nl	nucleolus
FV	food vacuole	NP	nuclear pore
G	Golgi apparatus	NS	neurosecretory granule
GC	ganglion cell	O	operculum
G1	gland cell	P	nerve cell process (neurite)
Gly	glycogen	PV	pinocytotic vesicle
I	interstitial cell	S	secretory droplet
ICS	intracellular space	SC	sensory cell
ICW	invaginated capsular wall	SCb	supporting rod of cnidoblast
M	mitochondrion	SCn	supporting rod of cnidocil
Me	mesoglea	Sp	spines
Mf	myofibrils	St	stylets
MG	mucous gland cell	T	thread (filament)
MP	muscular process		
MT	microtubule		

Fig. 1 Apical surface of an epitheliomuscular cell. Two layers of material cover the plasma membrane: a thin homogeneous layer of low density and a thicker layer of finely fibrillar and granular material. Small vesicles are present immediately beneath the plasma membrane. A few dense mucus granules are present. Mitochondria and intracellular spaces are also present near the surface. 19,000X



Fig. 2 Apical portion of an epitheliomuscular cell. A layer of fibrillar material covers the plasma membrane. Numerous vacuoles, mitochondria, and intracellular spaces are present near the surface. Complex Golgi complexes are located in the cytoplasm. 29,000X

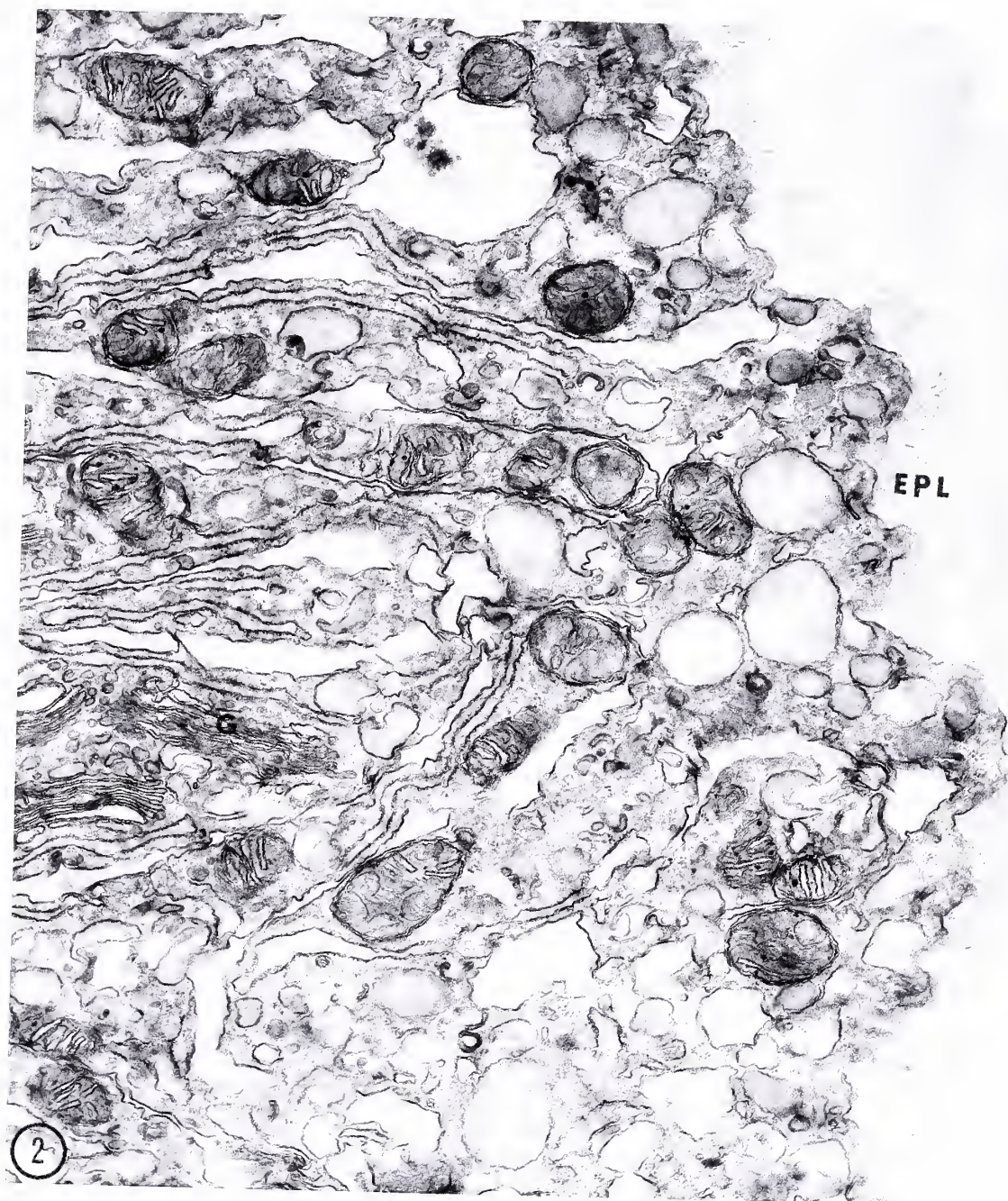


fig. 3 Apical region of two epitheliomuscular cells. A thick layer of fibrillar material and a thin clear homogeneous layer cover the plasm membrane. Numerous vacuoles, mitochondria, and intracellular spaces are present in the cytoplasm. Note that the adjacent cell membranes are thickened at one point (arrow) and an intercellular space lies a short distance below the thickening. 29,000X

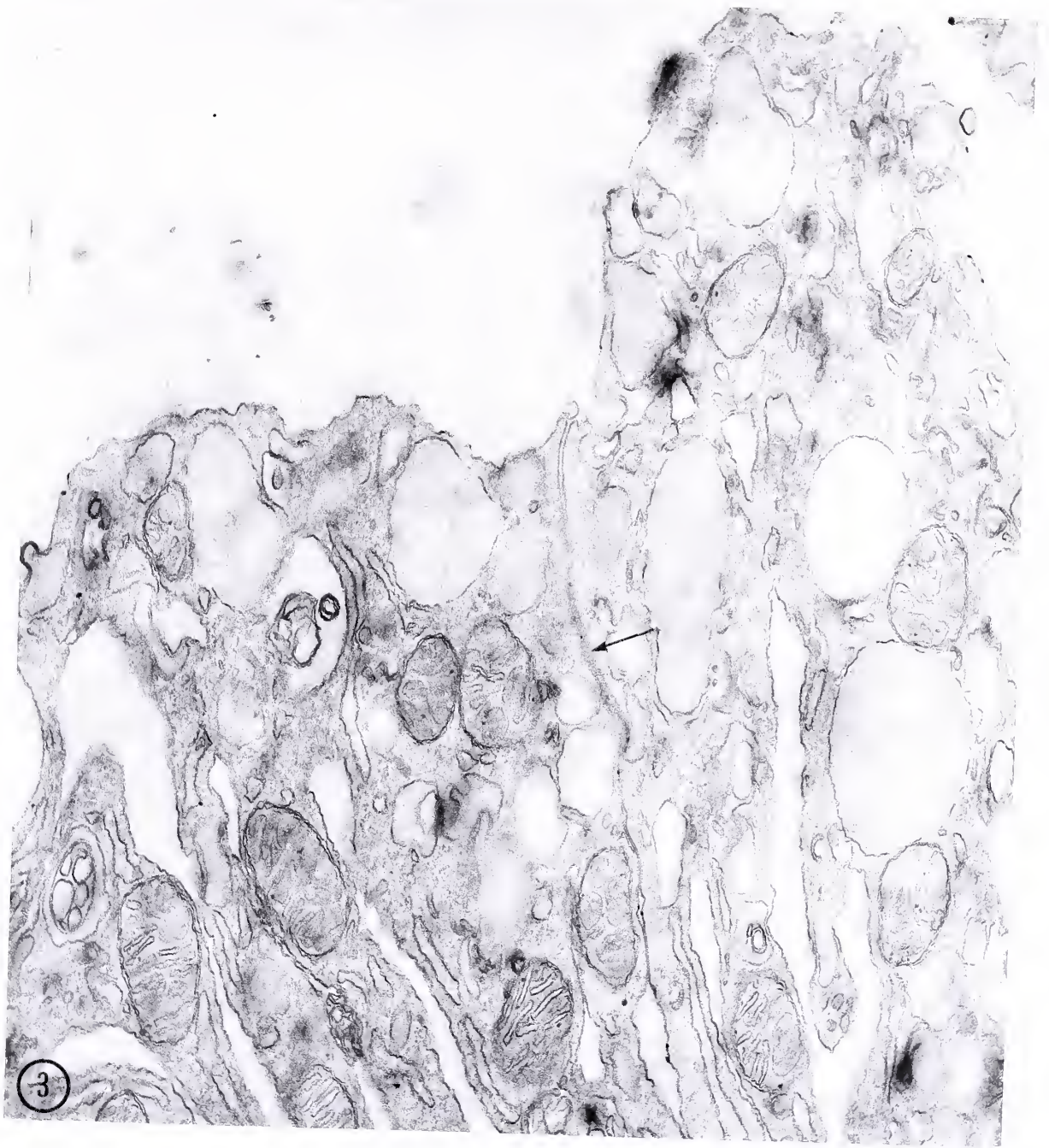


Fig. 4 Junction of two epitheliomuscular cells. Transverse bars (arrow) extend across the intercellular space near the surface of the cells. Two tongues of cytoplasm extend into the intercellular space below the desmosome. Finely granular and fibrillar material covers the cell surfaces. 88,000X

EPL

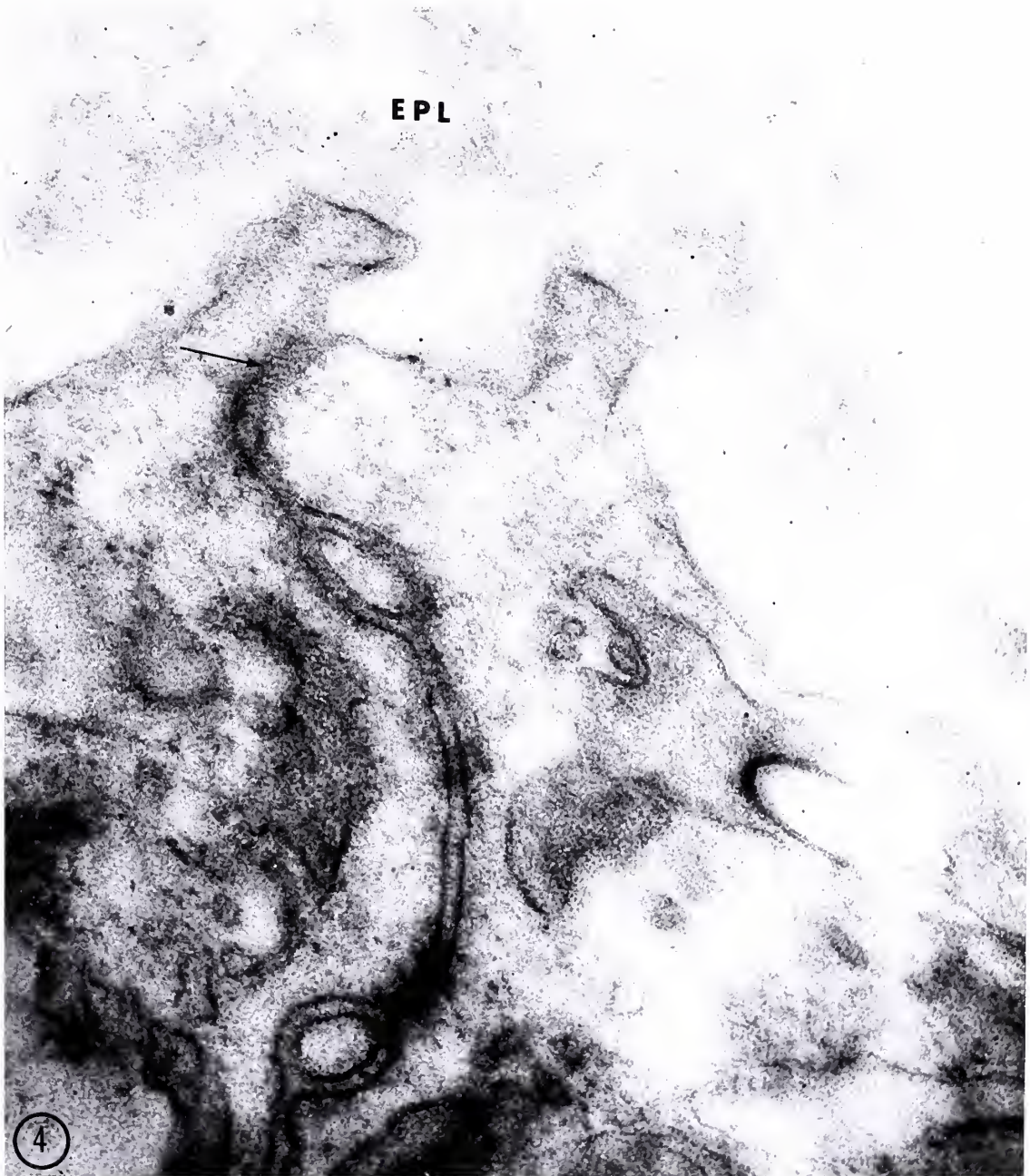


Fig. 5 Junction of two epitheliomuscular cells.
Note the thickenings of adjacent cell membranes.
A layer of low density lies above the plasma membrane
and is covered by a thick layer of finely granular
material. Large intracellular spaces are present.
65,000X

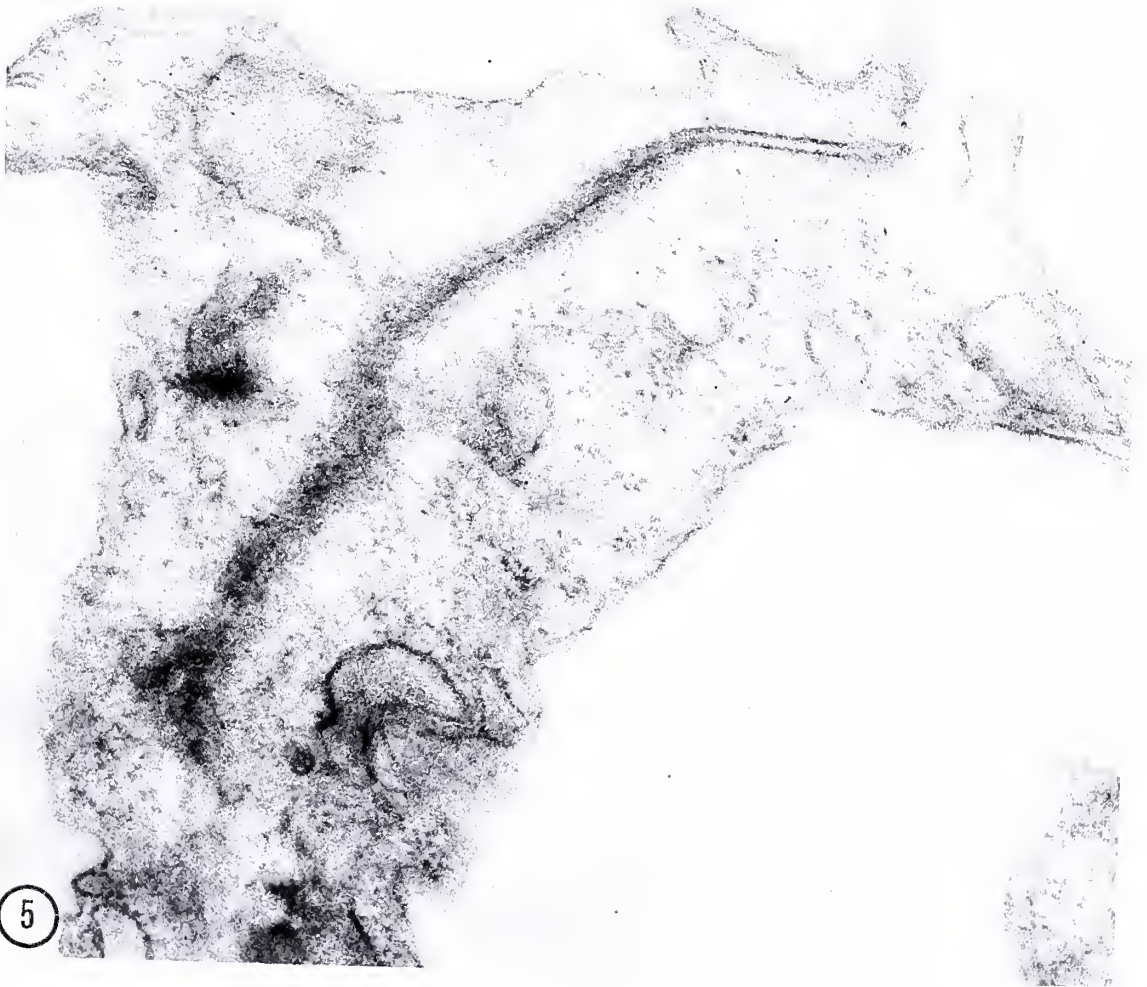


Fig. 6 Two adjacent epitheliomuscular cells. Note the separation of adjacent cell membranes to form an intercellular space. An intracellular space, large vacuoles, mitochondria, and a few ribosomes are present in the cytoplasm. 65,000X

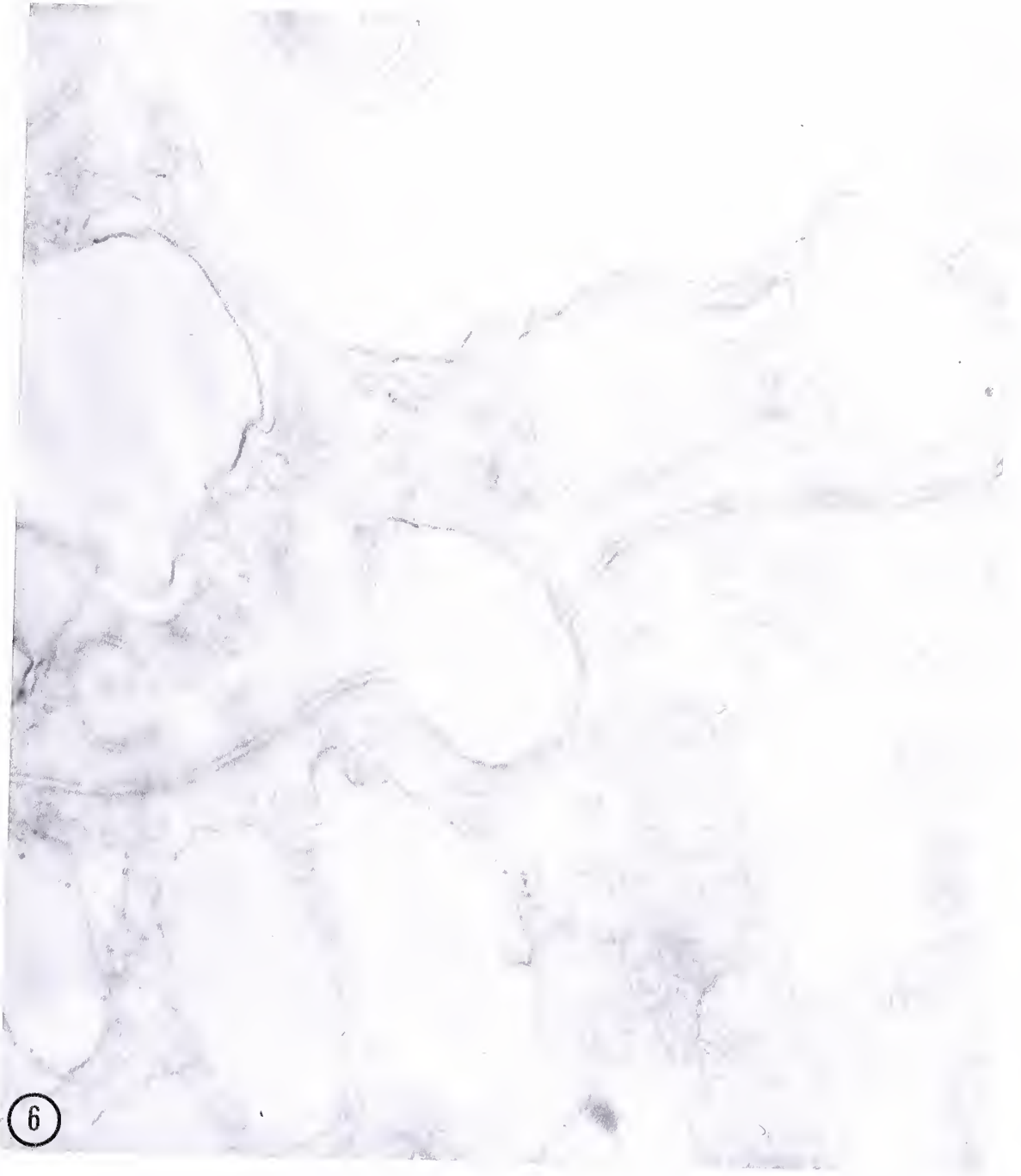


Fig. 7 Surface of a digestive cell. A fibrillar feltwork covers the plasma membrane. Microvilli extend into the digestive cavity. Dense granules, presumably glycogen, are present in the cytoplasm. Note the tubular structures (arrows) which contain transverse bars and a central core. 65,000X

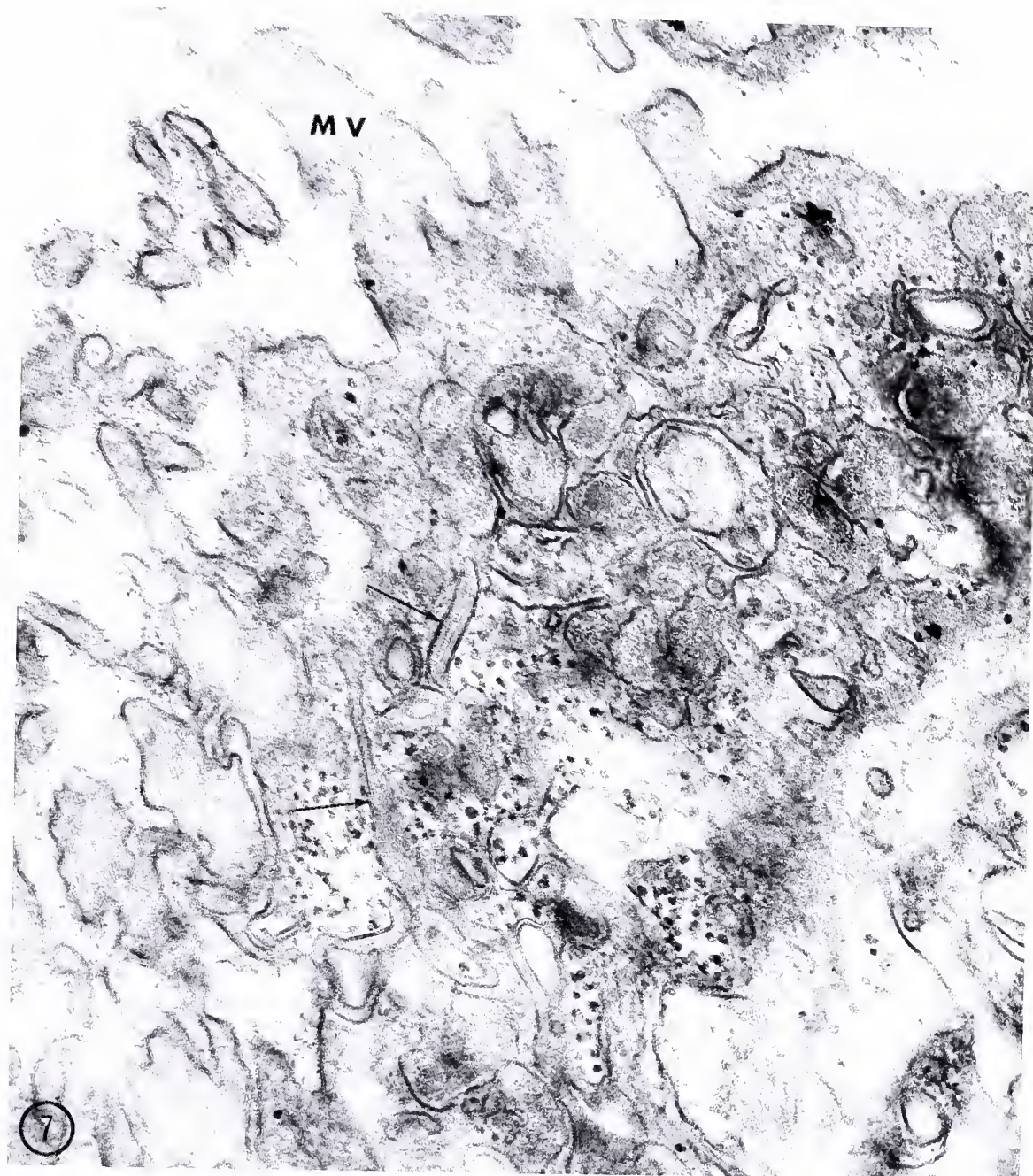


Fig. 8 Surface of a digestive cell. A fibrillar layer covers the plasma membrane. A few vesicles are present beneath the plasma membrane. Pinocytotic channels are present and some enter a large membrane bound space deeper in the cytoplasm. 65,000X

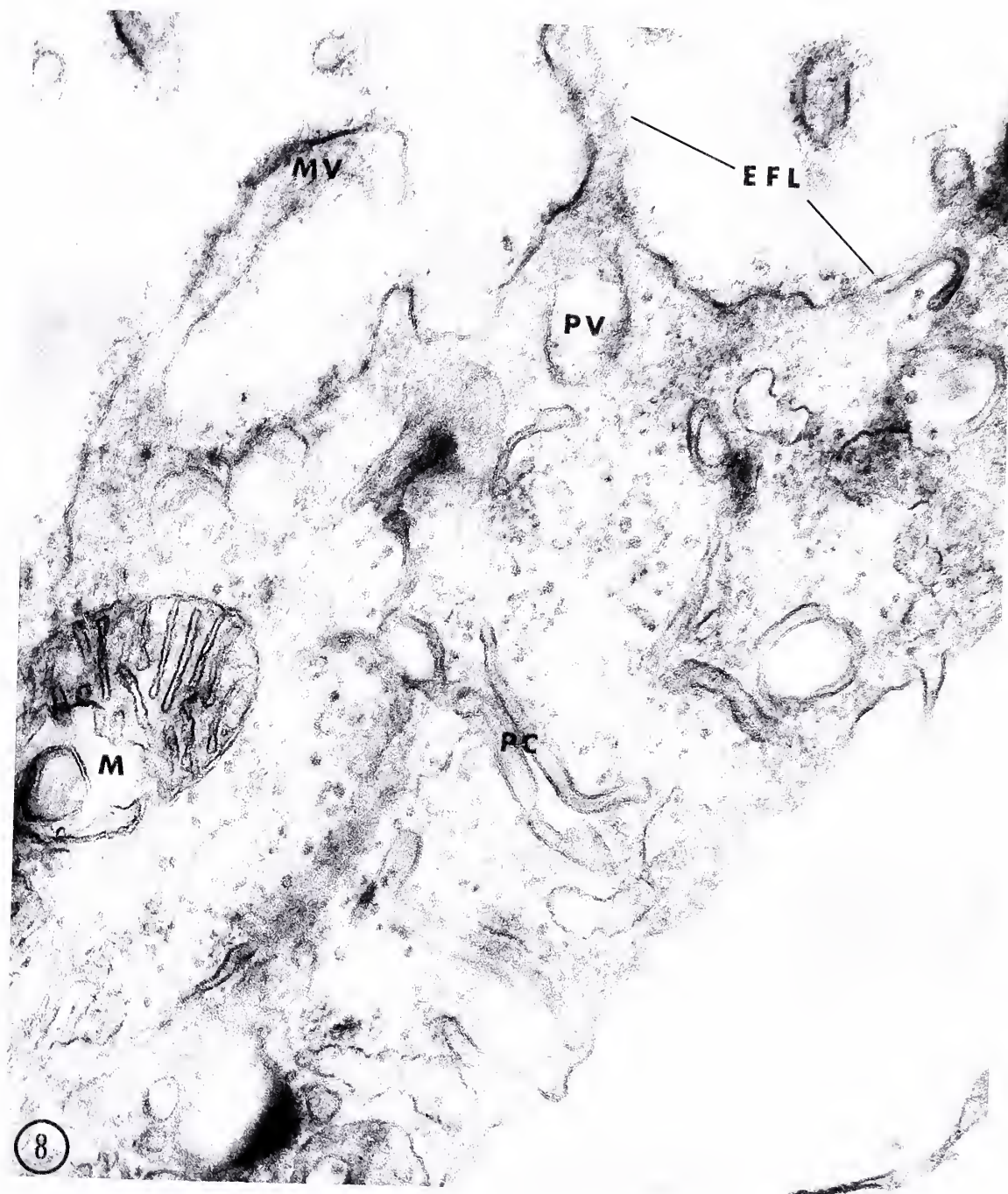


Fig. 9 Apex of a digestive cell. A microvillus and two flagella extend into the digestive cavity. A fibrillar felt-work covers the plasma membrane. Granules, pinocytotic channels, and mitochondria are present in the cytoplasm. 65,000X

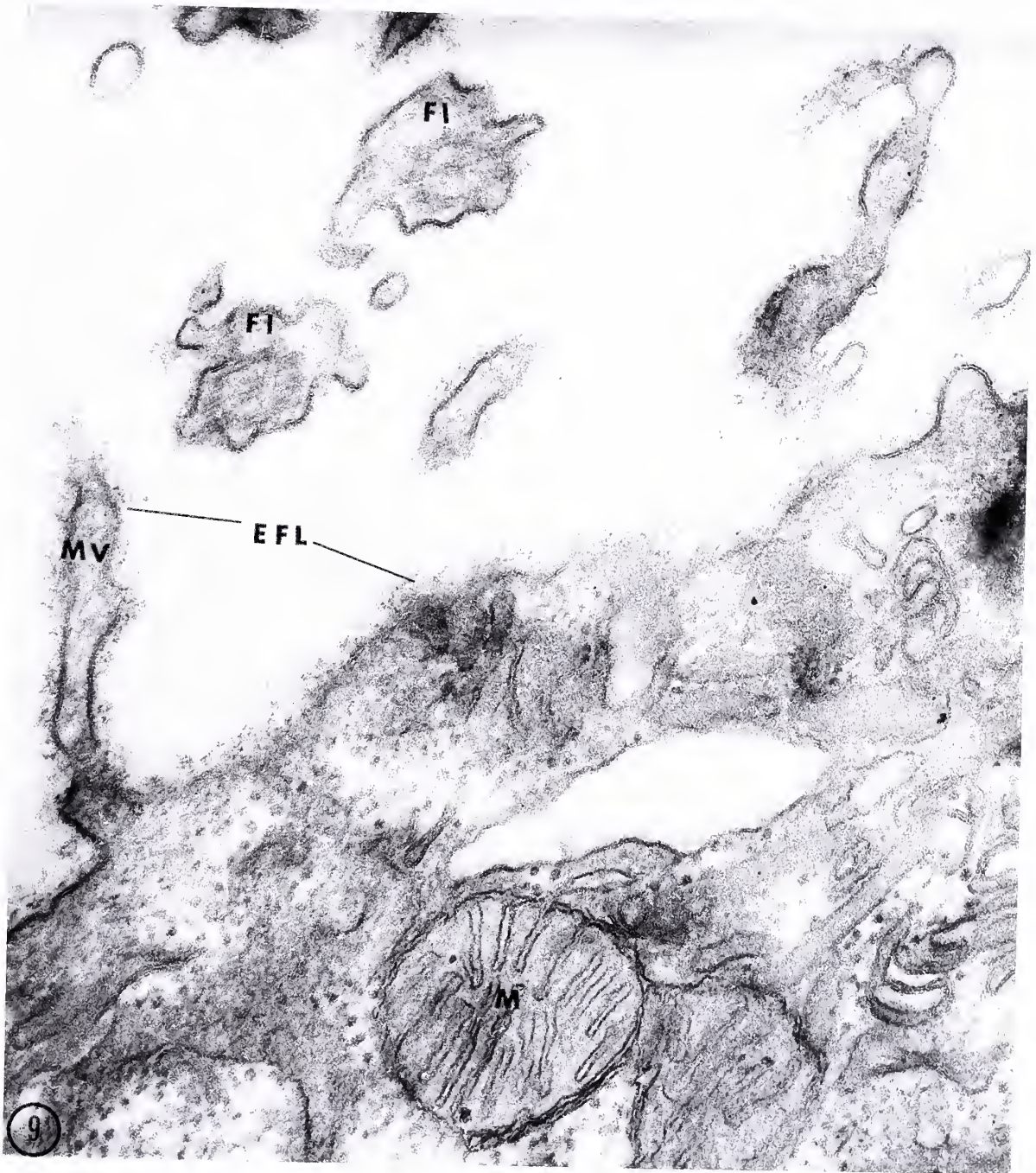


Fig. 10 Surface of a digestive cell. A flagellum is present in longitudinal section. Note the cytoplasmic vesicles and mitochondria. Fibrillar material covers the plasma membrane which consists of two thin dense layers separated by a space of low density (unit membrane). 65,000X

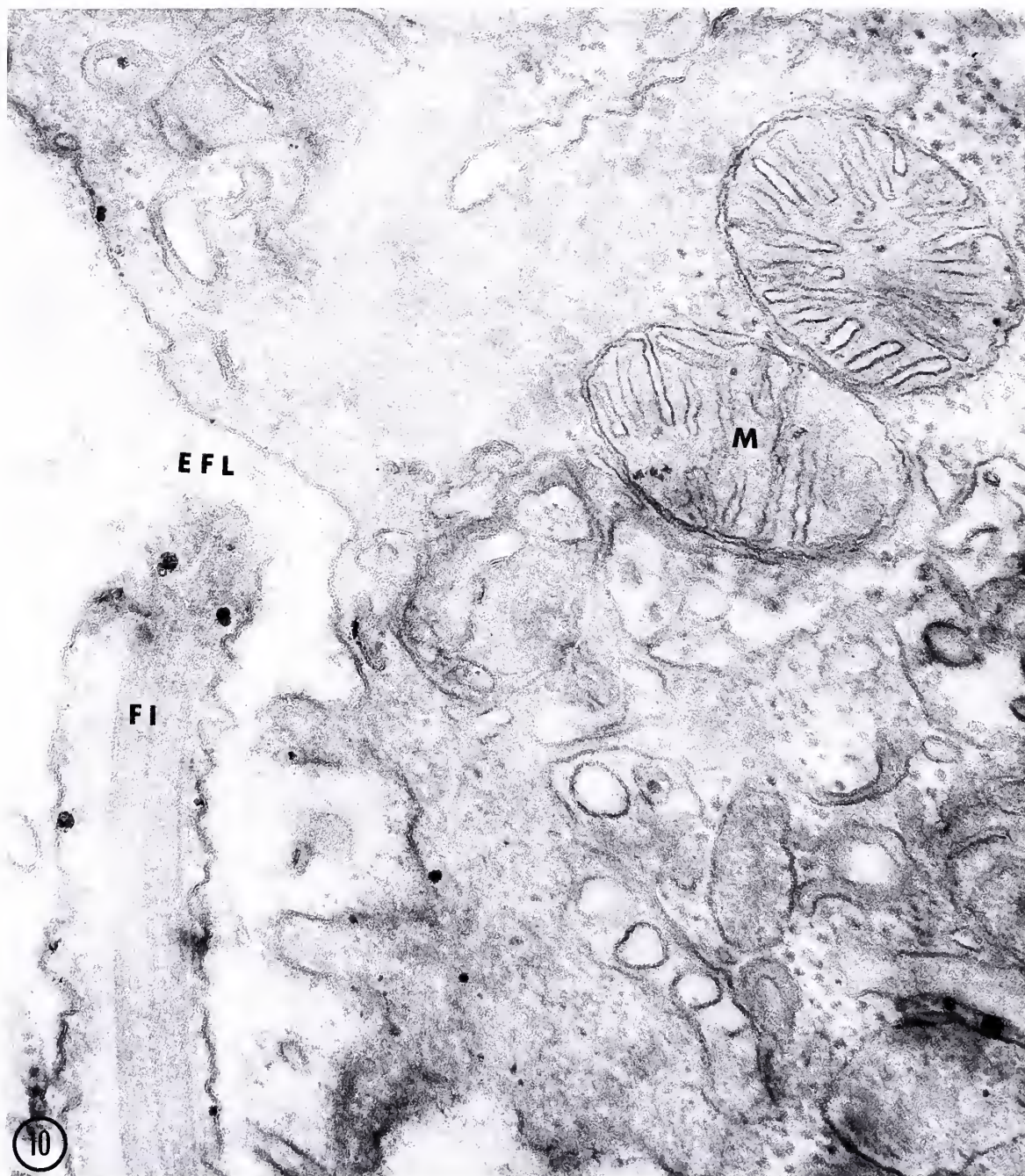


Fig. 11 Digestive cavity and surface of two digestive cells. Note the numerous flagella within the digestive cavity. The flagella contain nine outer double rods surrounding two inner rods. The plasma membranes are covered with fibrillar material. 29,000X

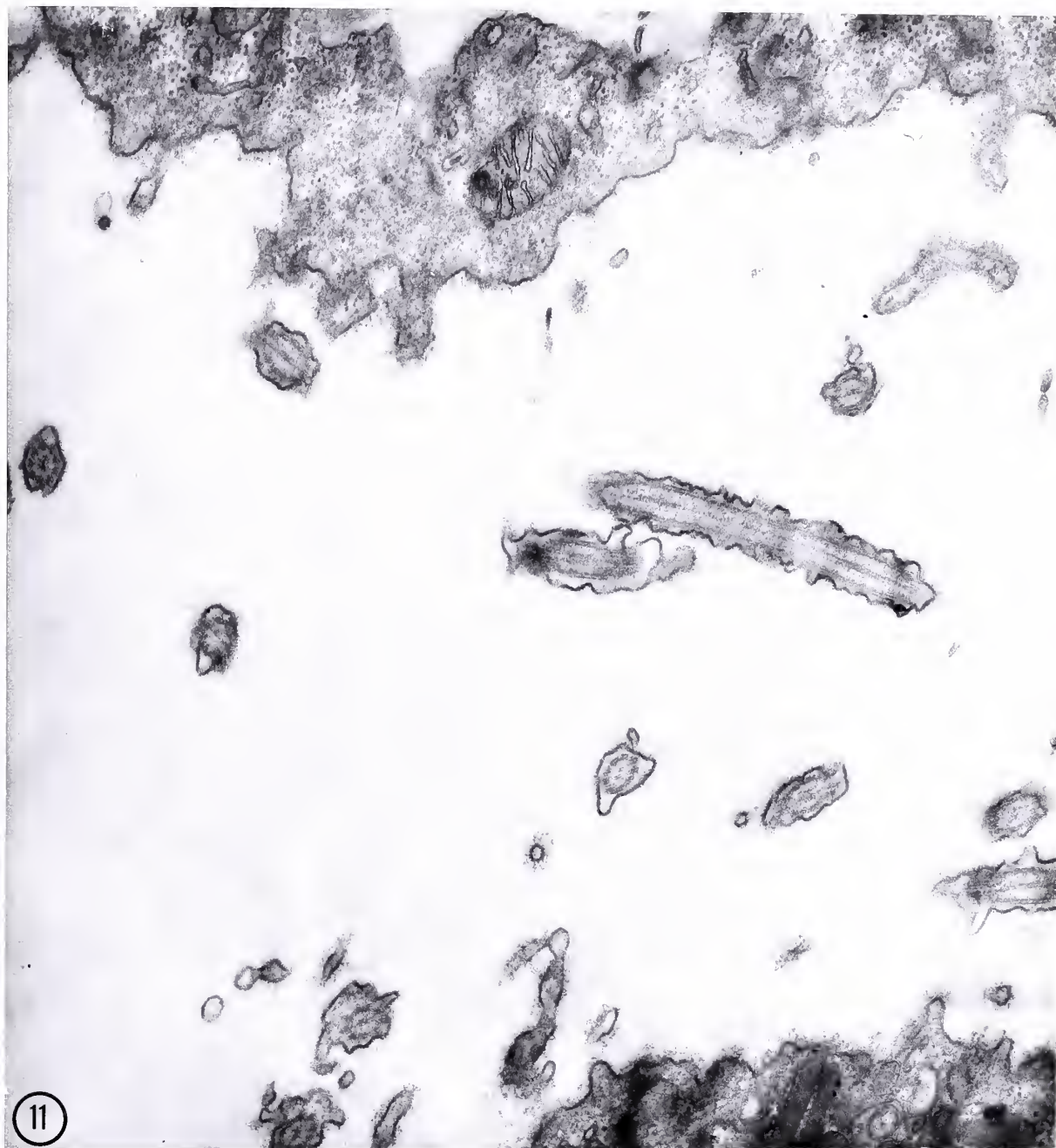


Fig. 12 Apical portions of two digestive cells. Microvilli extend into the digestive cavity. A fibrillar felt-work covers the plasma membranes. Flagella are present near the surface. Many small dense granules, presumably glycogen, are present in the cytoplasm and within membrane bound vacuoles. A space is formed by the divergence of adjacent plasma membranes. Transverse bars extend across the intercellular space at one point. Striations are present beneath the plasma membrane of one cell (arrow). A large mitochondrion is present near the surface. 65,000X

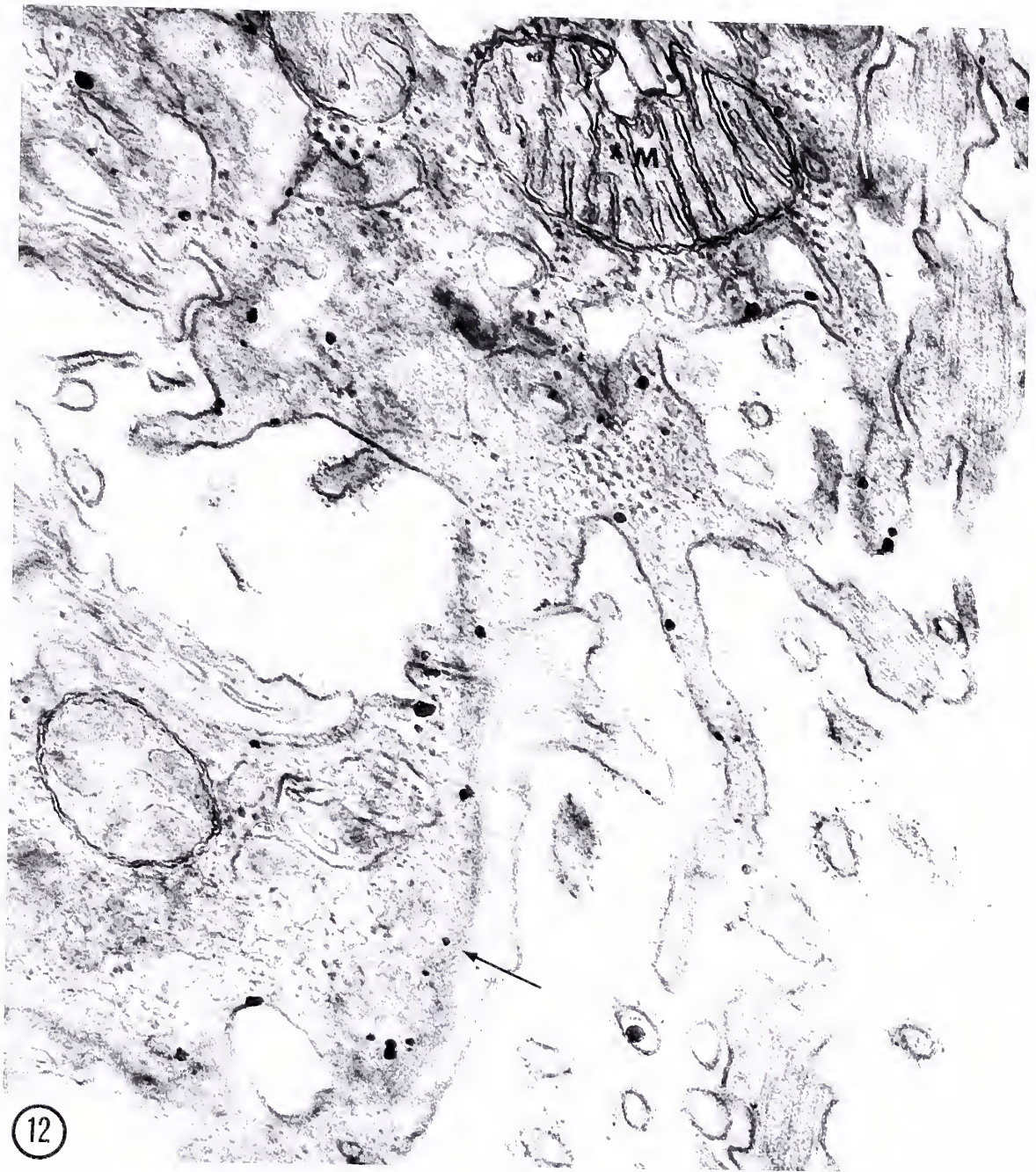


Fig. 13 Epidermal surface after 20 minutes exposure to ferritin. Note that ferritin particles are attached and embedded in the fibrillar layer covering the plasma membrane but none is present within the cytoplasm.

78,000X



Fig. 14 The hydra has been immersed in ferritin for 20 minutes. Note that slender cytoplasmic processes of an epitheliomuscular cell have extended outward to enclose ferritin particles.

78,000X

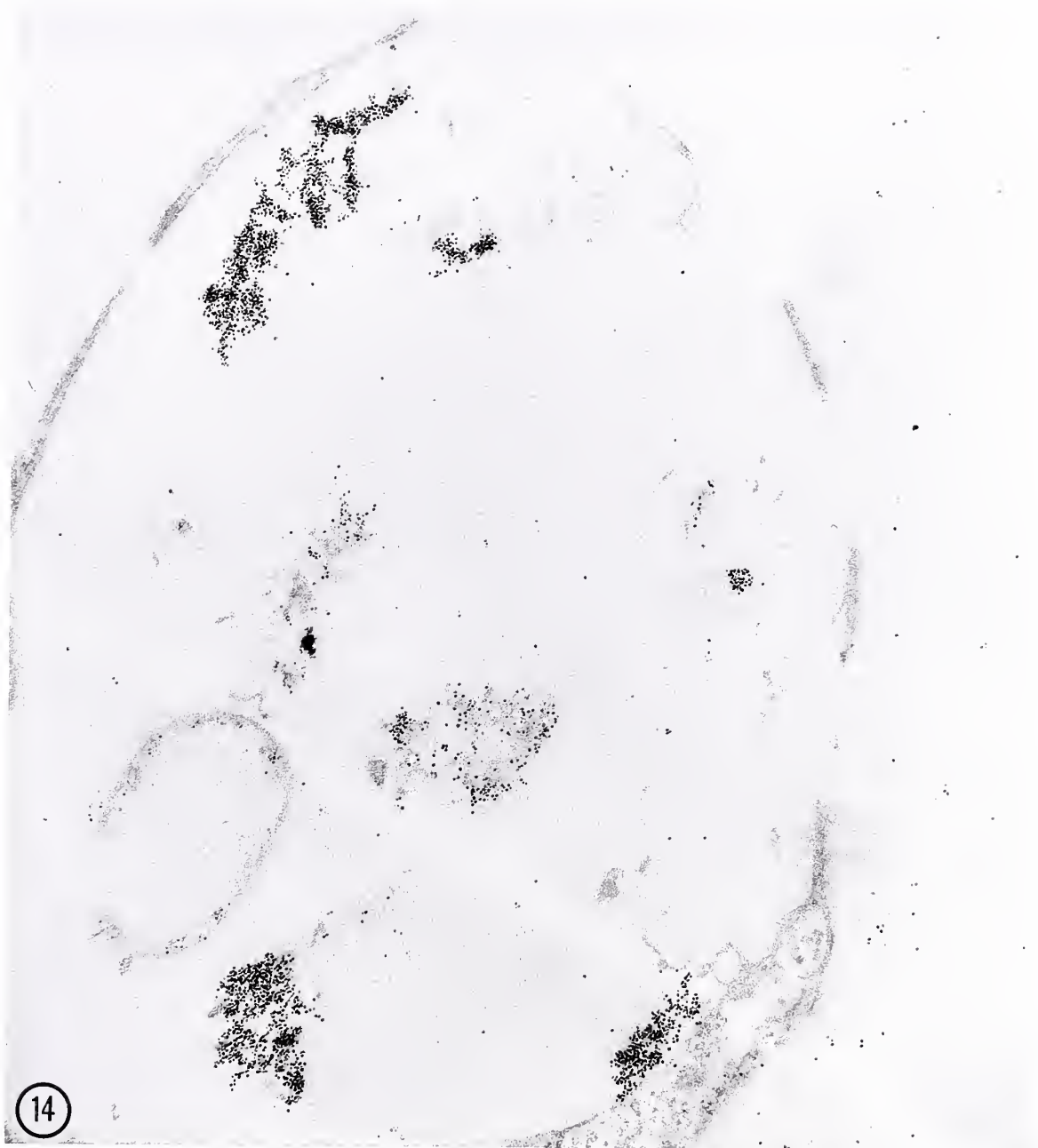


Fig. 15 Epidermal surface after 45 minutes exposure to ferritin. Note that ferritin is present in the large membrane-bound cell fragment which has pinched off from the epidermis. No ferritin is present within the epidermis proper. A few ferritin particles are attached to the fibrillar layer coating the epidermis (arrows). 78,000X

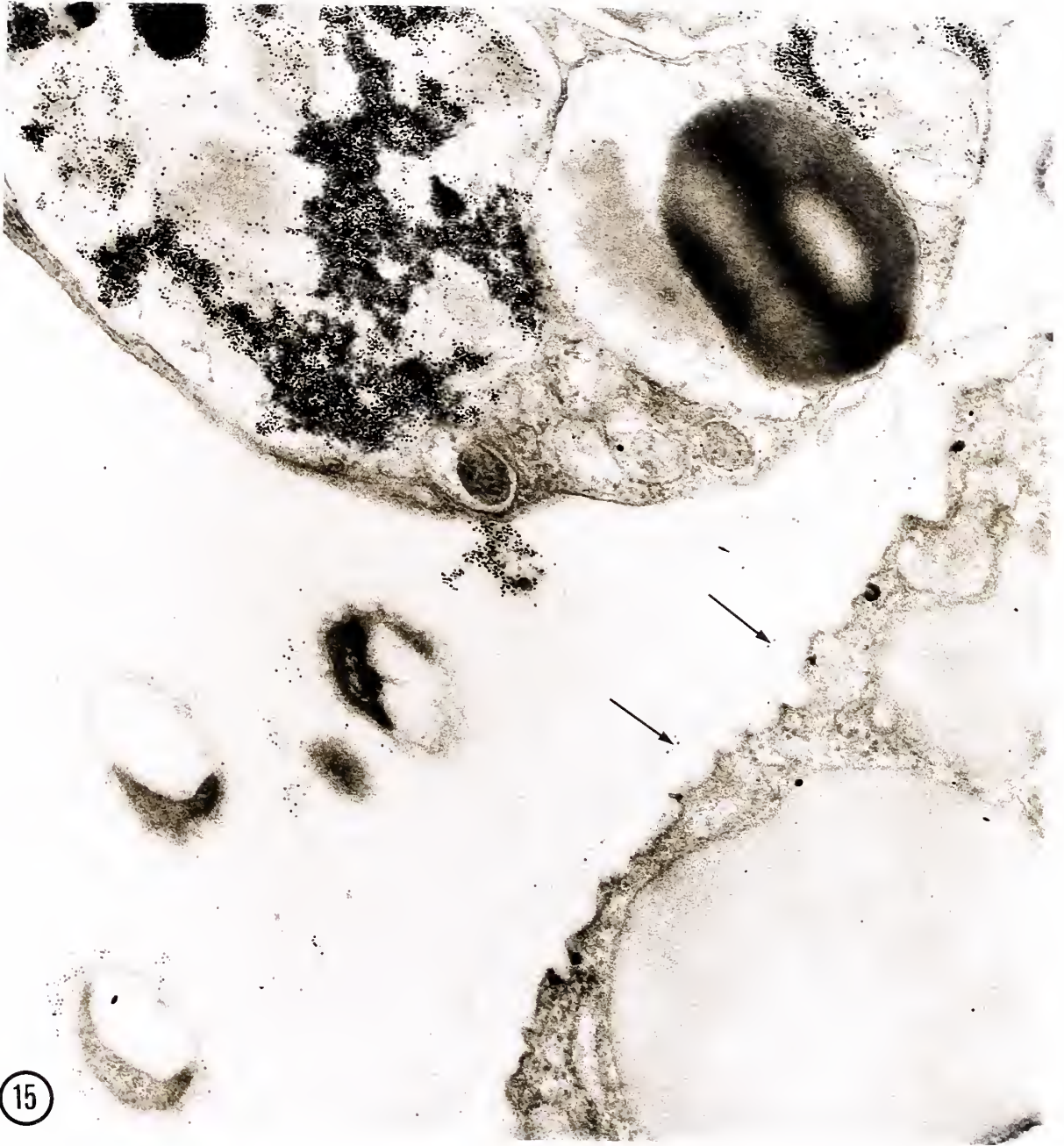


Fig. 16 Digestive cell 20 minutes after injection of ferritin into the digestive cavity. Ferritin is attached to the fibrillar coating of the plasma membrane. Pinocytotic invaginations at the bases of microvilli contain ferritin. A large membrane bound accumulation of ferritin is present deeper in the cytoplasm. 29,000X

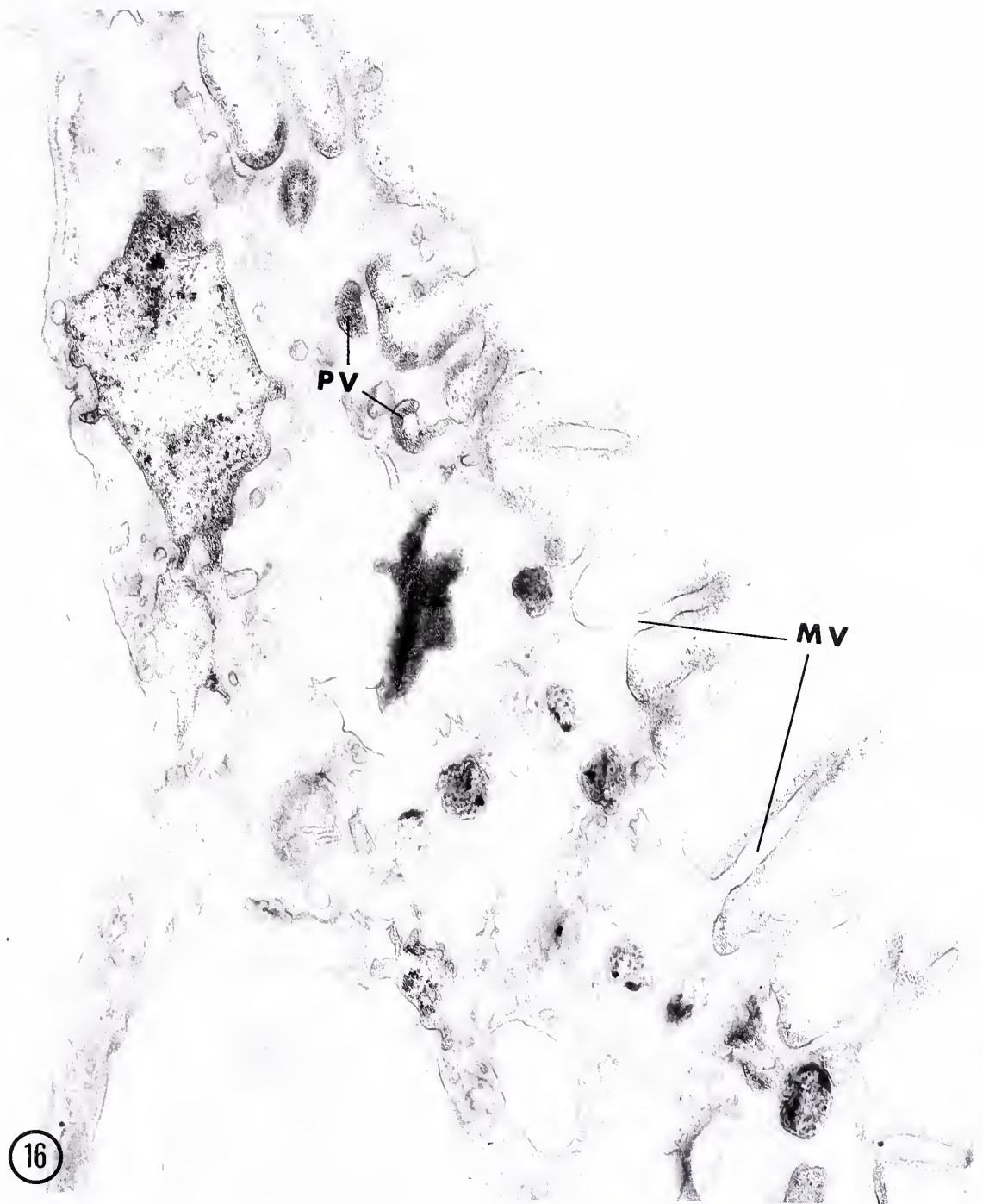


Fig. 17 Surface of digestive cells 20 minutes after injection of ferritin into the digestive cavity. Note that ferritin is adherent to the fibrillar layer covering the plasma membranes. Ferritin is present within invaginations at the bases of the microvilli. Also note the presence of ferritin within cytoplasmic channels. 29,000X

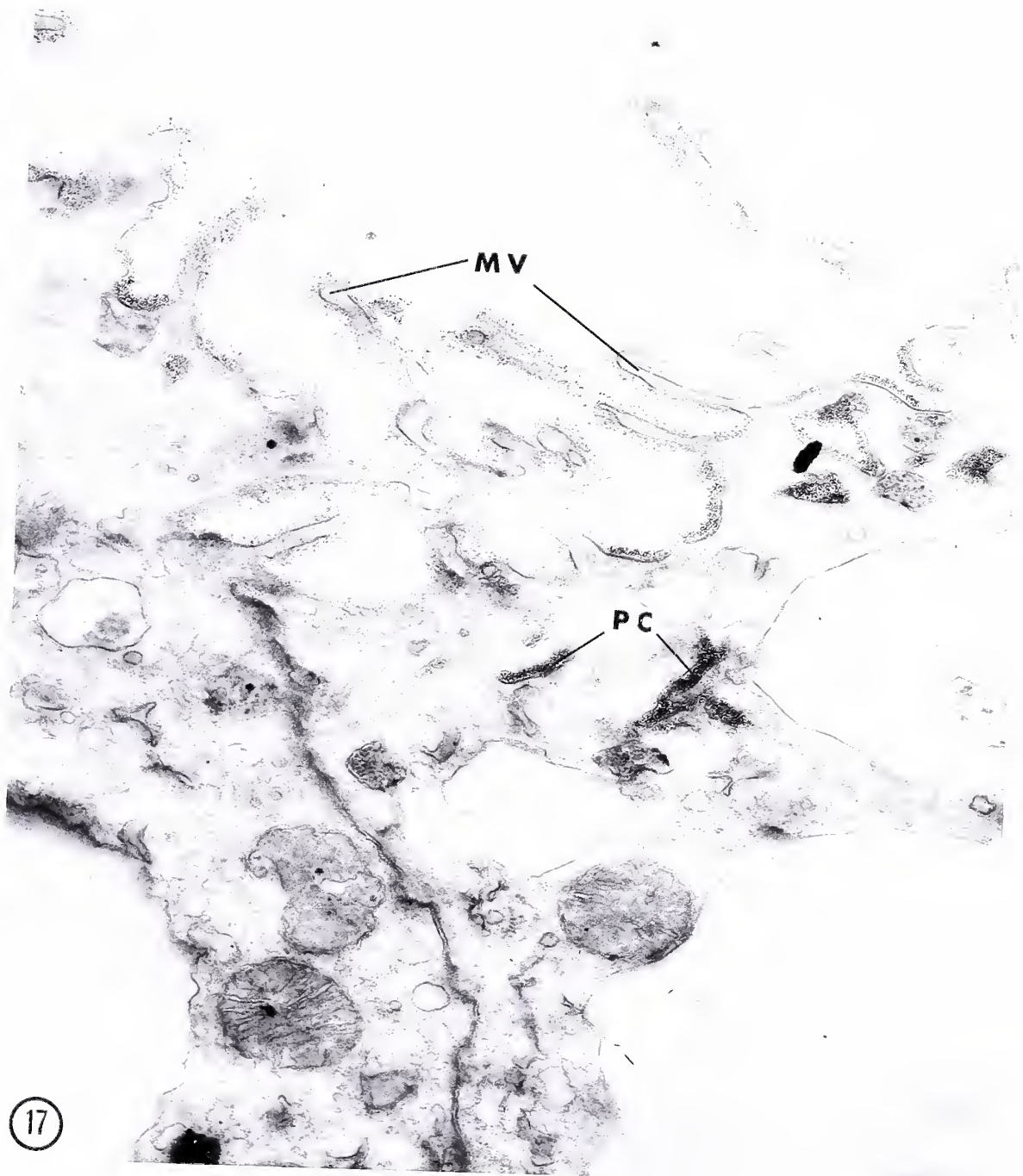


Fig. 18 Apical region of a digestive cell 45 minutes after introduction of ferritin into the digestive cavity. Note the presence of ferritin in small pinocytotic channels (arrows). Large amounts of ferritin are densely packed within a membrane bound space. Very little ferritin is attached to the surface and pinocytotic vesicles are not present at this time (compare with figs. 16 and 17). 78,000X

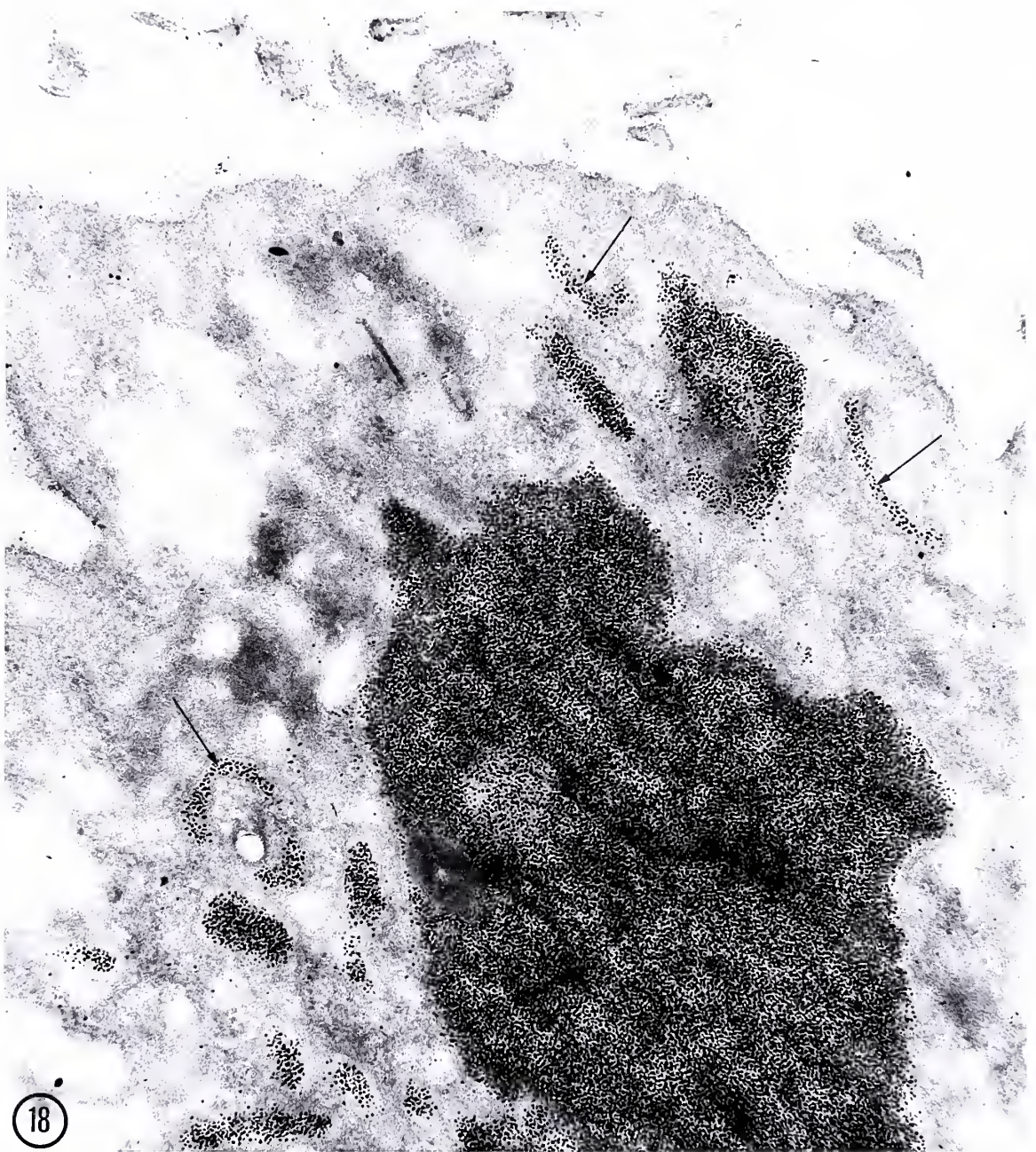


Fig. 19 Gastrodermis after 45 minutes exposure to ferritin. Ferritin is present within cytoplasmic channels. Two channels enter the large dense accumulation of ferritin (arrows). Note that ferritin is not present on the cell membrane or within pinocytotic invaginations. 78,000X

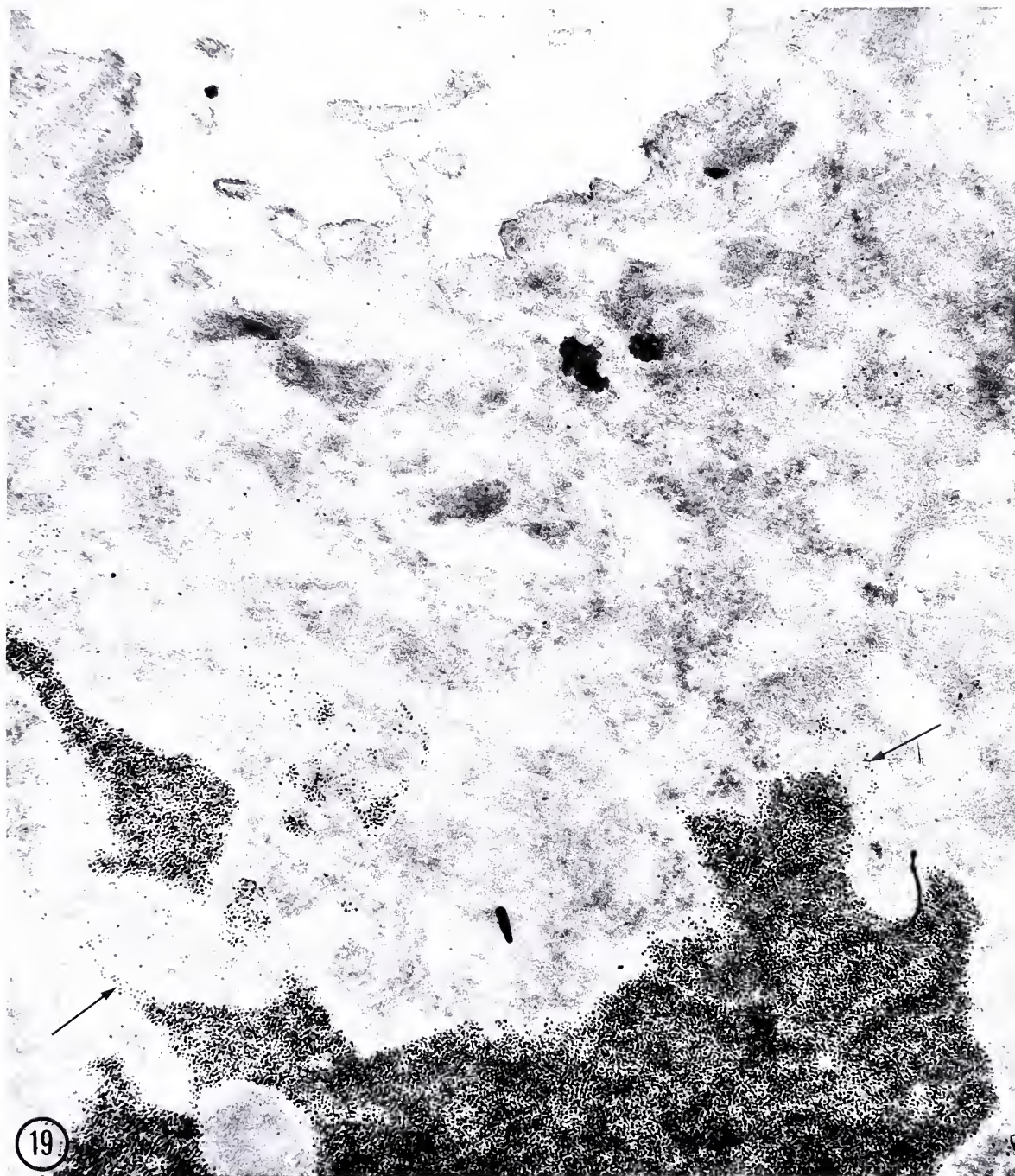


Fig. 20 Surface of a digestive cell which has been exposed to ferritin and cysteine ($10^{-3}M$). Practically no ferritin is present within the cell. A few ferritin particles are attached to the fibrillar coating of the plasma membrane. 52,000X

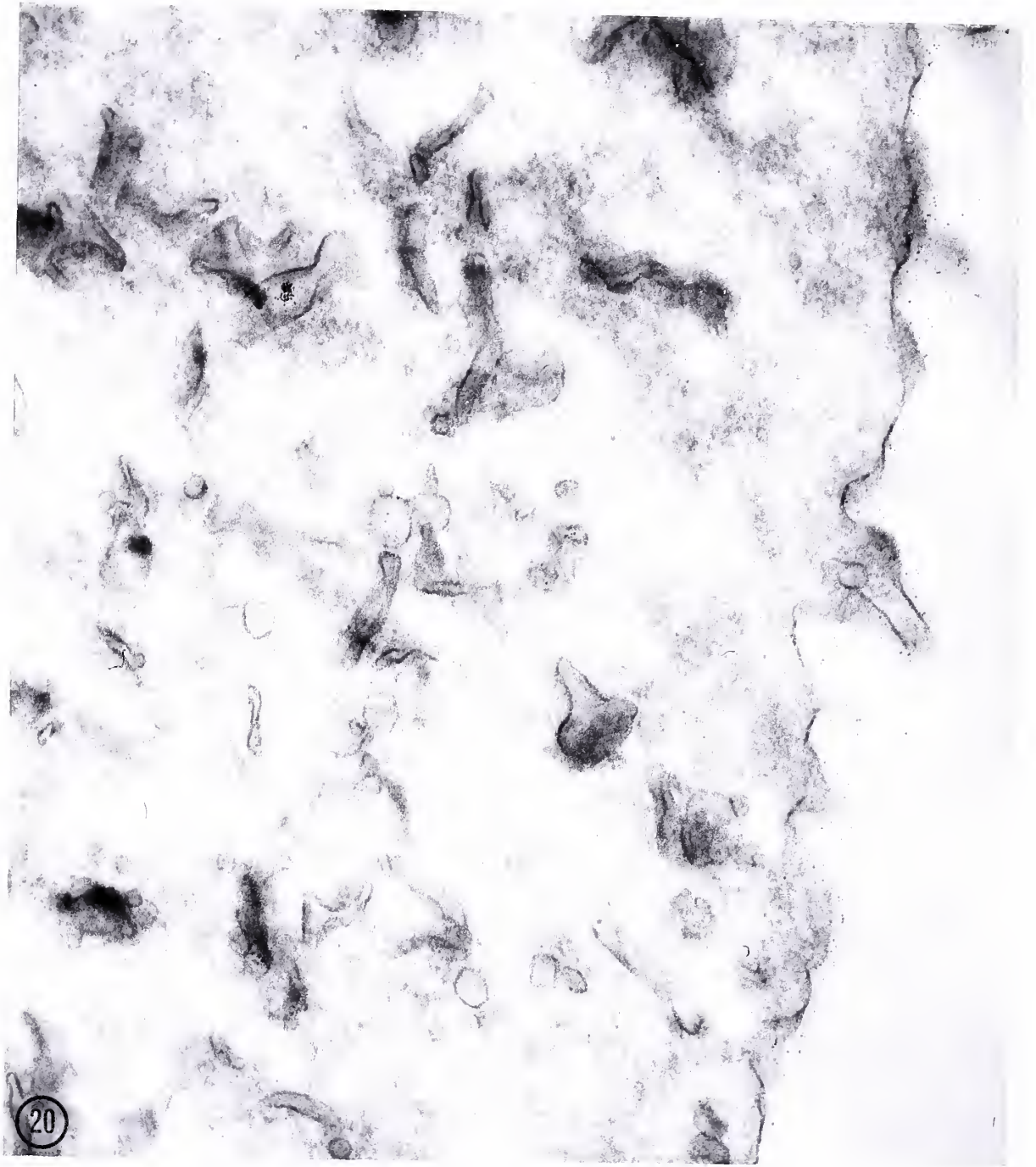


Fig. 21 Digestive cell exposed to ferritin in the presence of NaF (10^{-3} M). Note the absence of ferritin within the cell. Some ferritin is attached to the cell surface and one pinocytotic channel contains ferritin. 29,000X

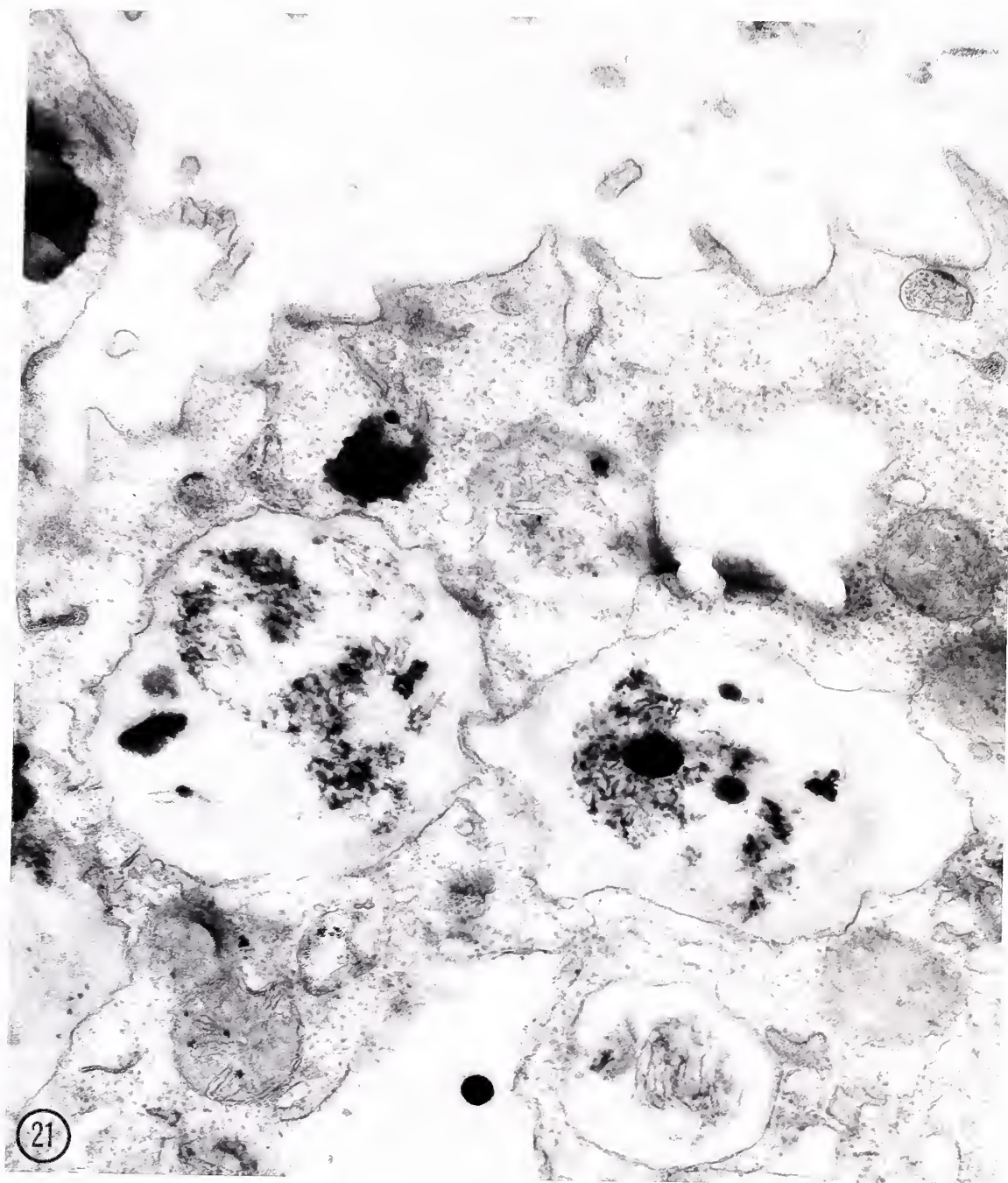


Fig. 22 Inhibition of ferritin uptake of digestive cells by phlorizin ($10^{-3}M$). Ferritin is attached to the fibrillar coating but only small amounts are present within the cell. Note the numerous dense lipid droplets. 15,000X

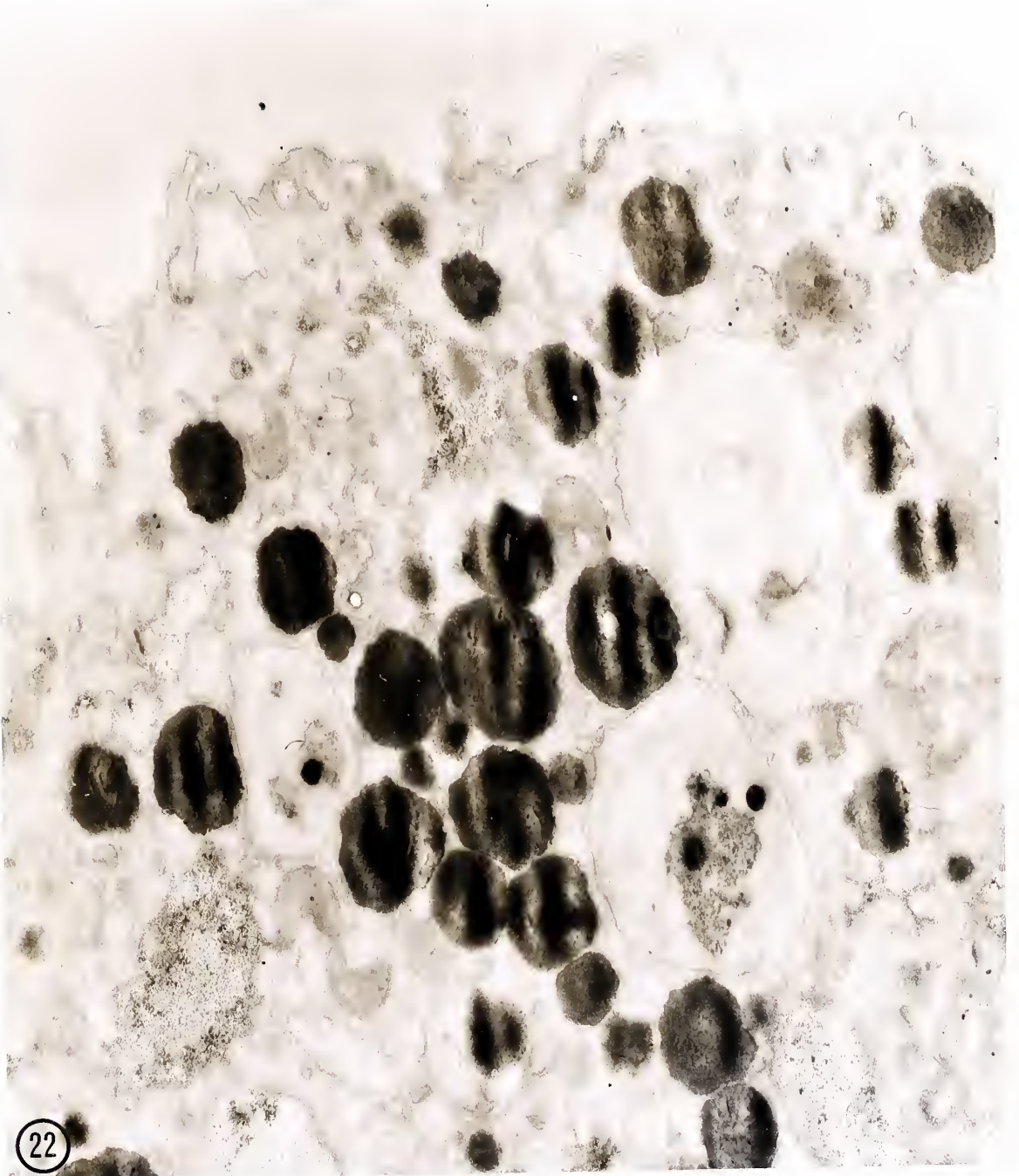


Fig. 23 Micrograph of gastrodermis treated with ferritin and physostigmine ($10^{-4}M$). Incorporation is uninhibited with ferritin present on the plasma membranes, in pinocytotic invaginations and channels, and within membrane bound spaces. 29,000x

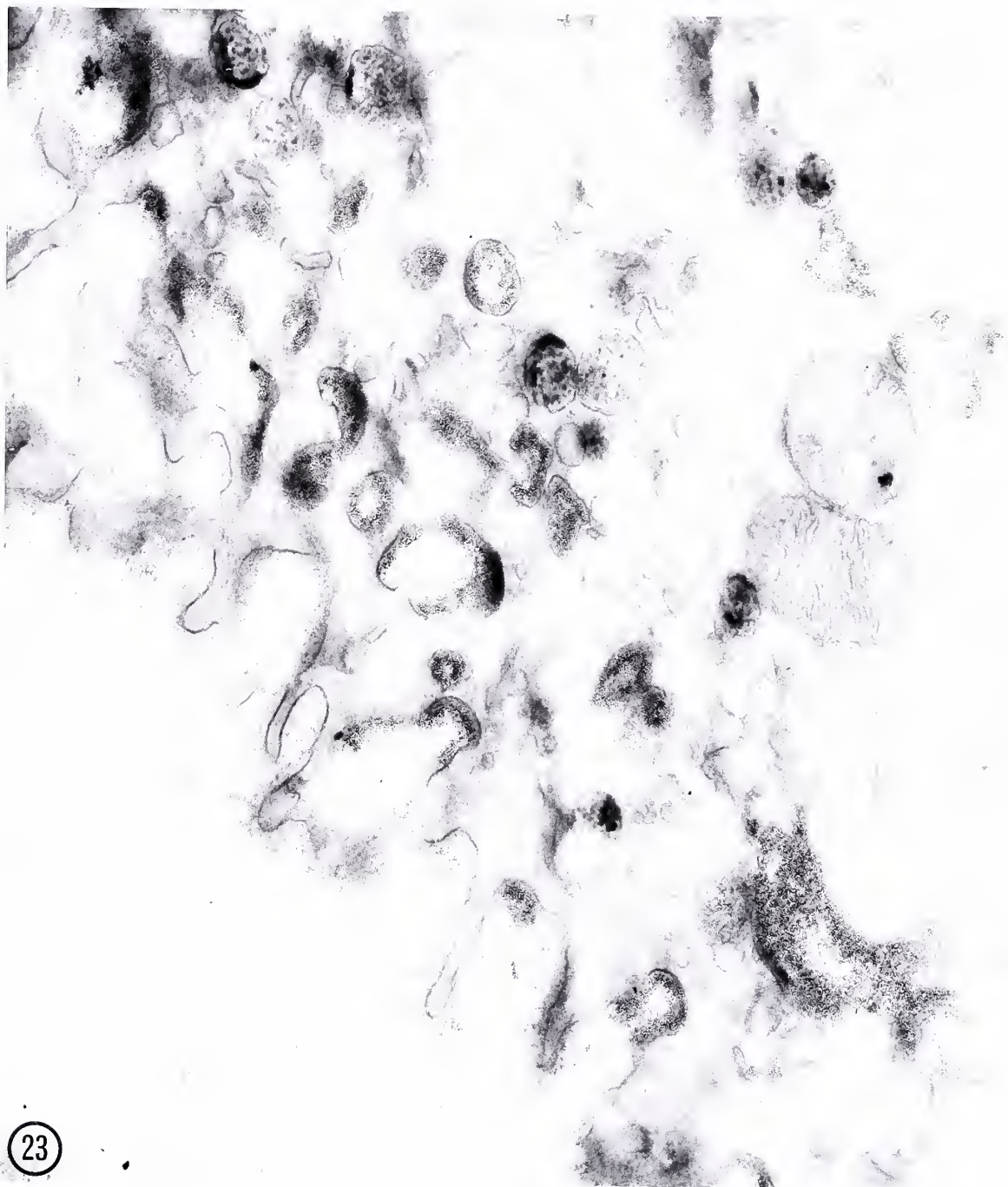
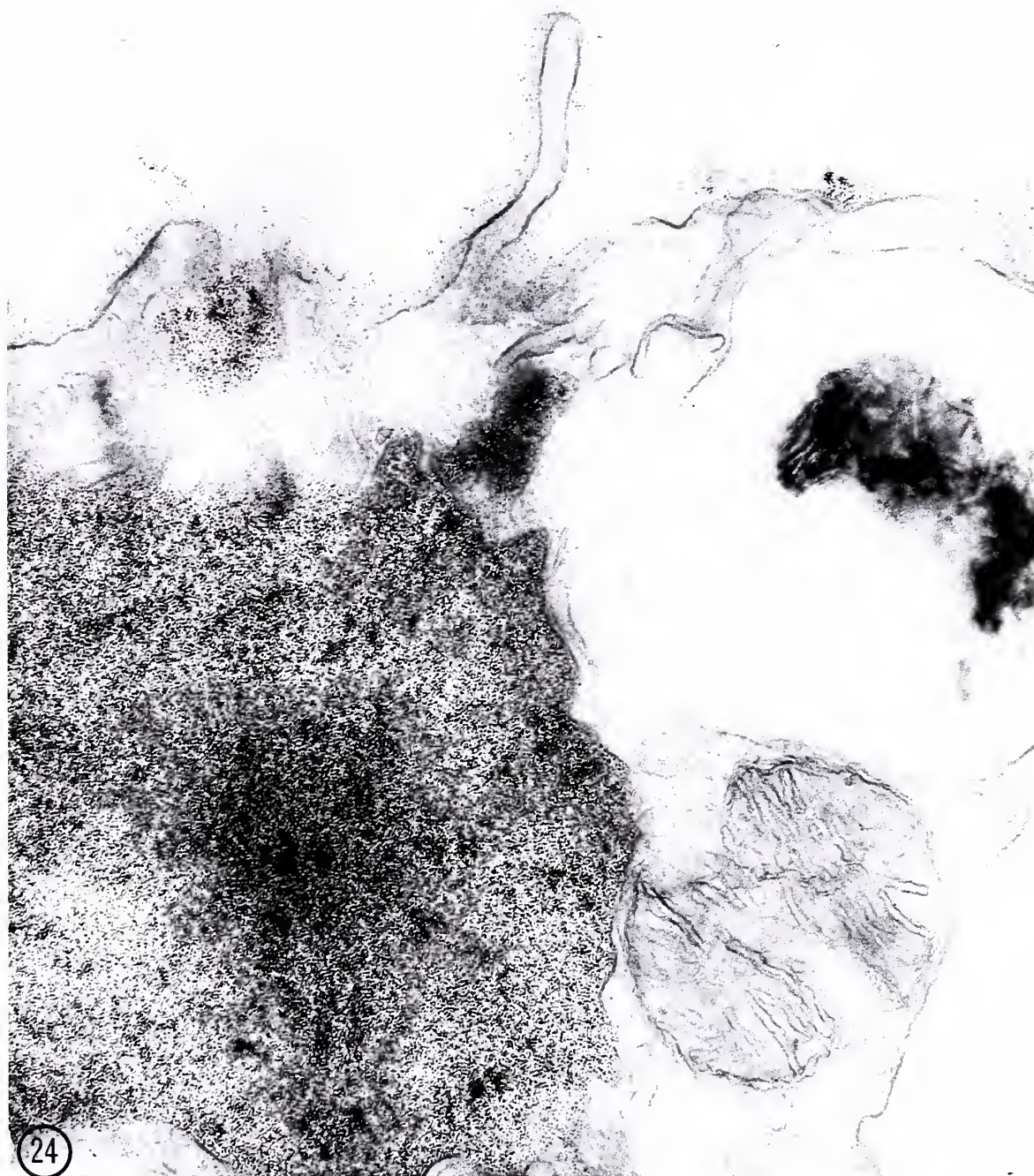


Fig. 24 Surface of a digestive cell 20 minutes after introduction of ferritin and sodium taurocholate ($10^{-2}M$) into the digestive cavity. A massive accumulation of ferritin is present in the cytoplasm. Ferritin is also attached to the cell surface and present within a pinocytotic invagination. 52,000X

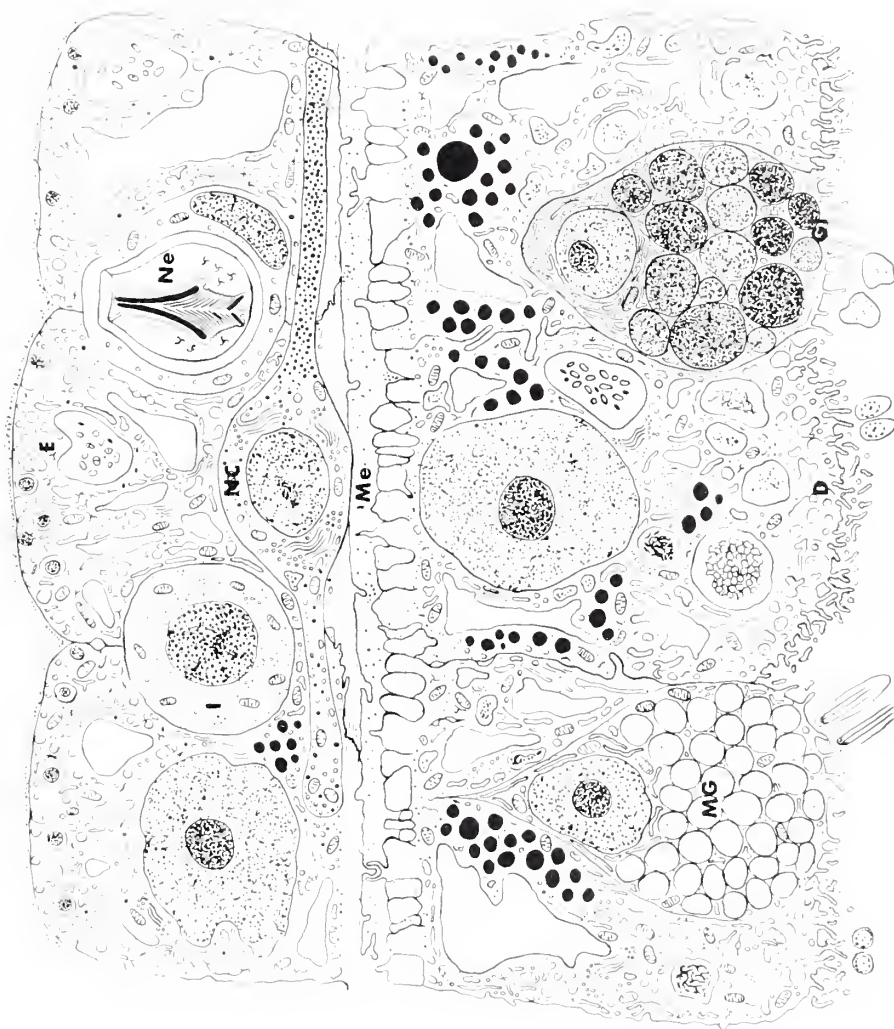


Conclusion

These studies have revealed the hydra to be remarkably specialized from a cytological and histochemical standpoint. Of particular interest were the cytological and regional enzymatic specializations, changes in enzymatic activity undergone during regeneration, and the relationship of enzymatic activity to permeability and other cell functions. Perhaps of greatest interest was the presence of neurosecretory material in a primitive nervous system and the apparent role of neurosecretion in regeneration, growth, and cell differentiation. The purpose of this final chapter is to reemphasize some of the findings of general biological significance applicable to all metazoan forms and to suggest future avenues of investigation. In the following paragraphs, the fine structure of hydra is reviewed with emphasis on the correlation of fine structure to cell function utilizing enzyme histochemical, pharmacological, and physiological techniques. These discussion are followed by a summary of the results concerned with growth and regeneration and the regulating influence of the nervous system in these processes.

The fine structural morphology of the six basic cell types in hydra is summarized in Text Figures 1 - 7. The interstitial cell was the least differentiated cell types and appeared to represent an embryonic reserve as

Text fig. 1 Diagram of a portion of the body wall of hydra. The epidermis is principally composed of cuboidal epitheliomuscular cells (E). Three other cell types are embedded in the epidermis: interstitial cells (I), cnidoblasts with their enclosed nematocysts (Ne), and nerve cells (NC). The mesoglea (Me) separates the epidermis from the inner gastrodermis which is chiefly composed of columnar digestive cells (D). Mucus gland cells (MG) and gland cells containing digestive enzymes (Gl) are embedded in the gastrodermis.

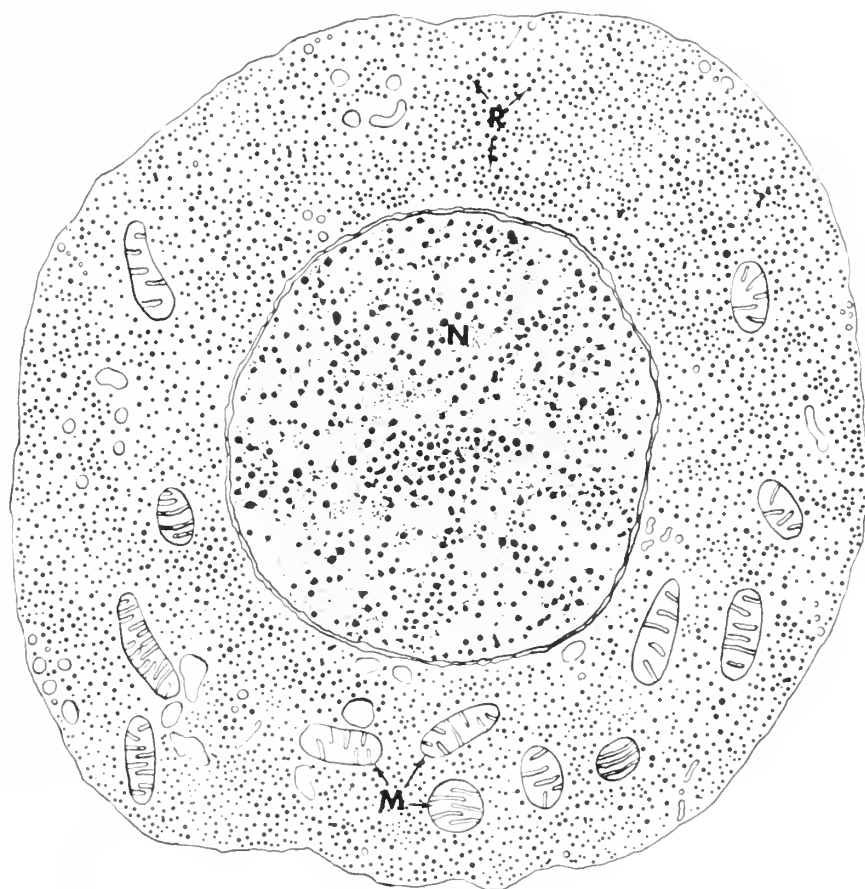


this cell was capable of differentiating into other cell types. Interstitial cells (Text Fig. 2) were characterized by an abundance of ribosomes and a paucity of other cytoplasmic organelles.

The differentiation of interstitial cells into cnidoblasts provided a model for the study of cell differentiation and the elaboration of a complex organelle. Nematocyst formation began within the Golgi apparatus and increase in size of the nematocyst was paralleled by the development of a complex lamellar rough-surfaced endoplasmic reticulum. Presumably, protein material elaborated by the endoplasmic reticulum was channeled through the Golgi complex and then deposited onto the nematocyst. Following attainment of full size of the nematocyst, the endoplasmic reticulum, Golgi apparatus, and nucleus regressed becoming inconspicuous.

The nematocyst (Text Fig. 3) was a remarkably complex organelle capable of discharging a thread which pierces the hydra's prey. A study of the factors regulating nematocyst discharge provided information concerning the control of effector cells. First, a number of enzymes, mainly phosphatases, were identified in the cnidocil and/or the capsule of mature nematocysts. Mechanical stimulation in the presence of enzyme substrates augmented nematocyst discharge whereas discharge was inhibited in the presence of enzyme inhibitors. These findings

Text fig. 2 Diagram of an interstitial cell. This undifferentiated cell contains a central nucleus (N) surrounded by a double membrane, mitochondria (M), a few cytoplasmic vacuoles, and numerous ribosomes (R). This cell is capable of differentiating into any other cell type.

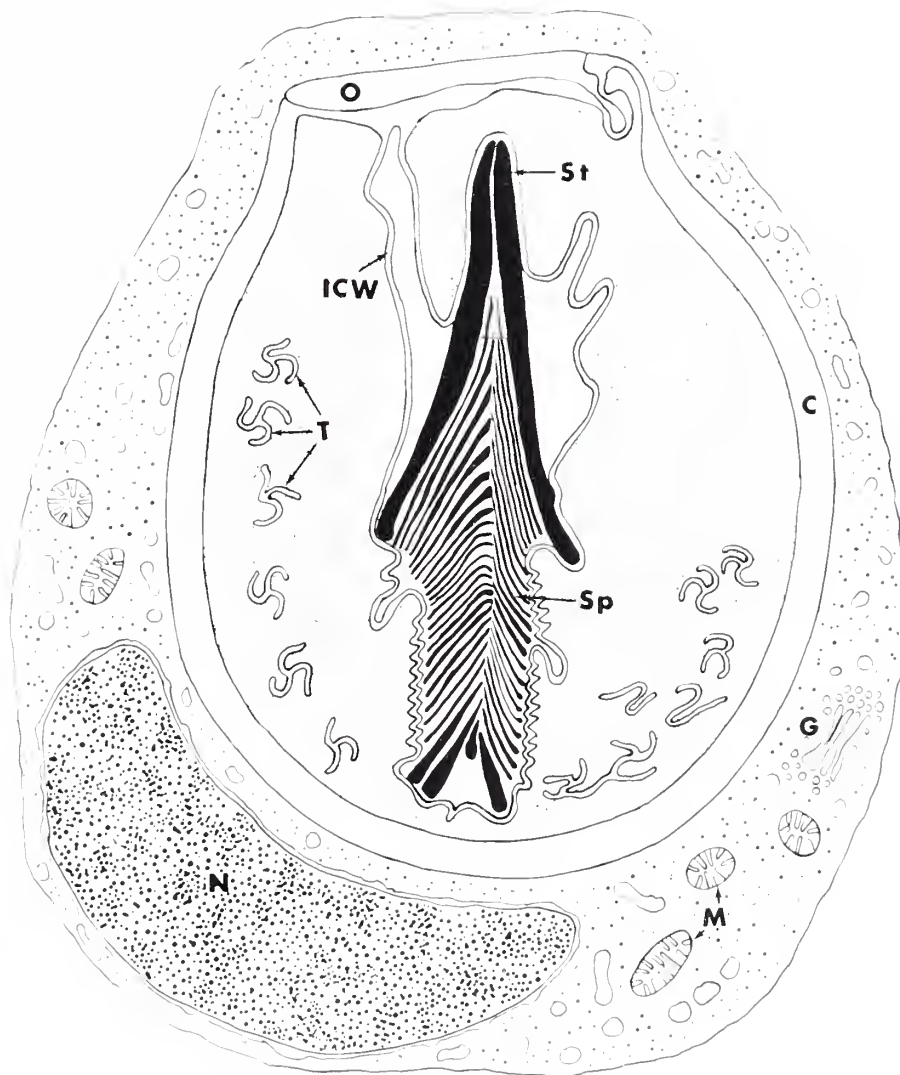


suggested that nematocysts containing strategically placed enzymes are able to respond in the presence of mechanical stimulation to chemical reagents which serve as enzyme substrates and therefore as effector substances. However, the nervous system also appeared to play some role in discharge since it was in structural contact with nematocysts and acetylcholine and some neurohumoral amines augmented discharge. Thus, both chemical and nervous stimulation in the presence of mechanical stimulation facilitate nematocyst discharge. The mechanism of ejection of the thread may be dependent on adenosinetriphosphatase activity since this enzyme is frequently associated with a variety of forms of motility in higher forms.

The fine structure of epitheliomuscular and digestive cells is diagrammed in Text Figures 5 and 6. Particular attention was paid to the surface specializations of these cells. The epidermal surface was covered by two layers, an inner thin homogeneous non-opaque layer and an outer thick granular and fibrillar layer. Digestive cells, on the other hand, possessed numerous microvilli extending into the digestive cavity. A loose feltwork of fibrillar material covered the plasma membranes. Pinocytotic invaginations were present at the bases of microvilli and membrane-bound cytoplasmic channels were present near the cell surface.

A study of ferritin uptake revealed the epidermal

Text fig. 3 Diagram of a cnidoblast with its enclosed nematocyst. The nematocyst consists of a capsule (C) surrounding the matrix and intracapsular structures. The stylets (St) and spines (Sp) are covered by the invaginated capsular wall (ICW). The thread (T) bears the shape of a three bladed propeller in cross section. The operculum (O) or lid is situated at the distal end of the nematocyst. The cnidoblast contains a nucleus (N), mitochondria (M), and a small Golgi apparatus (G).

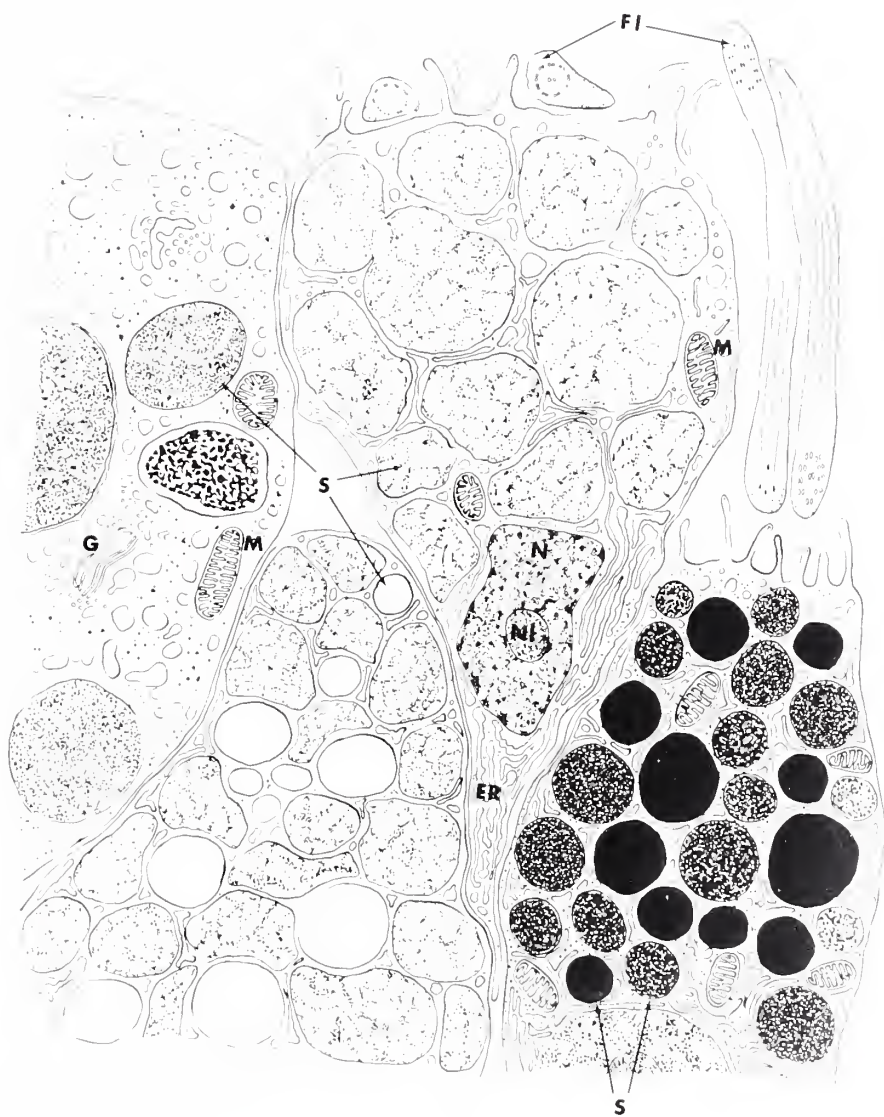


cells to be impermeable to ferritin whereas the digestive cells rapidly took up large quantities of ferritin. Ferritin particles were trapped first by the external fibrillar layer and then appeared in the pinocytotic invaginations at the bases of the microvilli. Particles were transported through the cytoplasm in pinocytotic channels and deposited within large intracellular vacuoles.

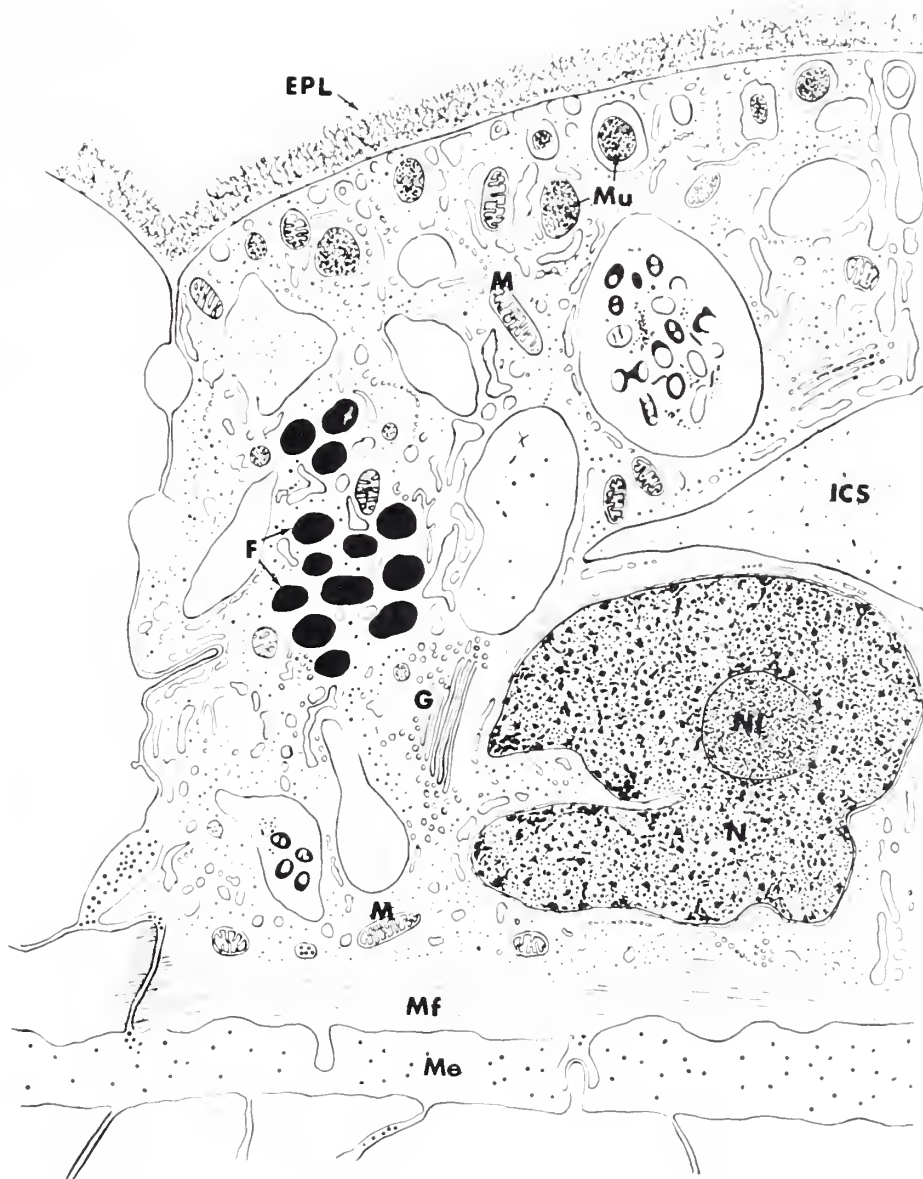
The presence of enzyme activities in or near the cell surfaces suggested that these may play a role in permeability. This suggestion was strengthened by the simultaneous occurrence of enzyme inhibition and inhibition of ferritin uptake. Particular attention was directed to adenosinetriphosphatase and it is hypothesized that the role of this enzyme in ferritin uptake relates to the synthesis of surface membrane via a phosphatidic acid mechanism. A study of this enzyme at the electron microscopic level should provide further information concerning the relationship of adenosinetriphosphatase to permeability and particularly to pinocytosis.

Neurons, of which there were two main types besides sensory cells, are described in Text Figure 7. Ganglion cells were characterized by a complex Golgi apparatus, cytoplasmic vesicles, ribosomes, and microtubules extending between the nucleus and the cytoplasm. Neurosecretory cells, on the other hand, were characterized by an abundance of dense granules in the cytoplasm and

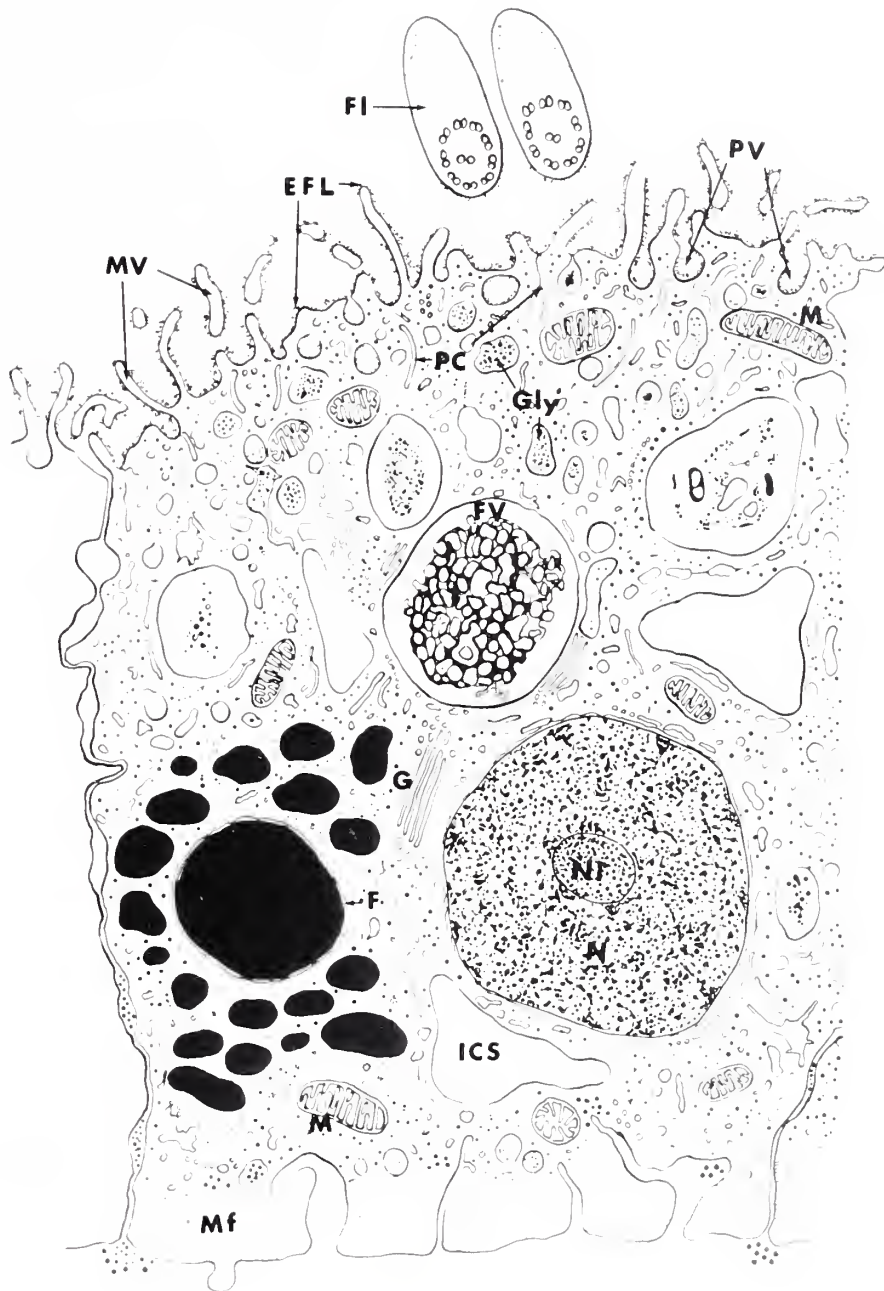
Text fig. 4 Diagram of the various types of gland cells in the gastrodermis. These cells possess microvilli, flagella(Fl), nuclei (N) with nucleoli (Nl), mitochondria (M), Golgi complexes (G), and a well developed endoplasmic reticulum (ER). The cells are filled with different types of secretory droplets (S). Some secretory droplets possess low density reticular or homogeneous contents while others contain a dense homogeneous or granular substance. The dense granules probably represent digestive enzymes while the less dense are presumably mucous droplets.



Text fig. 5 Diagram of a portion of an epithelio-muscular cell. The plasma membrane is covered with a thin layer of low density and the thick fibrillar external protective layer (EPL). The cell contains a large nucleus (N) with a nucleolus(N1), mucous (Mu) and lipid (F) droplets, mitochondria (M), a Golgi apparatus (G), and intracellular spaces (ICS). The base of the cell possesses muscular processes containing myofibrils (Mf). The mesoglea (Me) or supporting lamella lies beneath the muscular process. Dense granules are present in the mesoglea and inter-cellular spaces.



Text fig. 6 Portion of a digestive cell. This large cell contains a nucleus (N) with a nucleolus (Nl), lipid droplets (F), various sized vacuoles some containing ingested food (FV), intracellular spaces (ICS), mitochondria (M), and a Golgi apparatus (G). The surface of the cell is highly specialized with microvilli (Mv) extending into the digestive cavity, pinocytotic or phagocytic invaginations (PV) at the bases of the microvilli, and paired flagella (Fl). The surface membrane is coated by the external fibrillar layer (EFL). Pinocytotic channels (PC), small vesicles and vacuoles, and glycogen deposits (Gly) are located immediately beneath the cell membrane. The muscular processes containing myofibrils (Mf) at the base of the cell are seen in transverse section.



processes. The presumed role of these cells in the control of growth and differentiation is discussed below.

The distribution of enzyme activities of the mature hydra suggested that as cells undergo morphological and functional specialization they undergo enzymatic specialization. The cells of the tentacles, hypostome, and base contained the activities of several phosphatases while the intervening area (growth region, stomach, and budding region) were unspecialized enzymatically. Thus, as cell migrate distally and proximally from the growth region they acquired enzymatic specialization indicating that enzymatic differentiation parallels morphological differentiation. Similarly, when nematocysts have migrated to the tentacles and peduncle they acquire the activities of the special phosphatases and are then functionally fully developed and capable of discharging.

The notion that enzymatic specialization parallels morphological development was confirmed by a study of the changes in distribution of enzymatic activity in the regenerating hydra. Furthermore, the converse, i.e., as cells lose their functional specialization they lose enzymatic specialization, was found to be true since the initial stage of regeneration was characterized by a loss of enzymatic activity as cells changed from a normal situation to one in which rapid growth was the major expression. During the second stage of regeneration, that of active

Text fig. 7 Two types of nerve cells in hydra.

The cell on the right, the ganglion cell, is characterized by microtubules (MT) extending from the nucleus (N) into the cytoplasm and cell processes and by elaborate Golgi complexes (G). The latter are situated between the nucleus and cell process and are composed of flattened stacks of lamellae and numerous vesicles. Mitochondria (M) and ribosomes are also present in the cytoplasm of the cell and its process.

The second cell, the neurosecretory cell (left), is characterized by an abundance of small dense granules (NS) in the cytoplasm and cell process. This cell possesses a Golgi apparatus with dense granules in the dilated ends of the parallel lamellae. Mitochondria, ribosomes, and a few microtubules are also present in this cell type.



growth, the cells were unspecialized enzymatically, a situation similar to that occurring in the growth and budding regions of normal hydra and in small immature buds. The final stage of regeneration was that of enzyme differentiation in which specialized cells acquired enzymatic activity simultaneously with the appearance of functional capacities.

Furthermore, it appeared that the hydra possessed some system for the recognition of anatomical form. Anatomical incompleteness coincided with the first two stages of regeneration and was accompanied by growth and enzymatic unspecialization. The third stage of regeneration in which anatomical form was complete appeared to be recognized and was followed by enzymatic differentiation. The most likely means of disseminating the necessary information concerning anatomical completeness or incompleteness is the nervous system.

Direct implication of the nervous system in regulation of growth and form was obtained by a study of the nervous system in the regenerating hydra. When a hydra was transected, nerve cells at the regenerating site increased greatly in number by 12 hours of regeneration. Furthermore, a variety of neuropharmacological agents inhibited in varying degrees regeneration, attainment of normal form, and enzymatic differentiation of bisected hydra. The large increase in nerve cells at 12 hours

did not occur in the presence of the inhibitory neuropharmacological agents. These findings indicated that the nervous system regulated the differentiation and growth of specialized cells in the hydra and the attainment of normal anatomical form of the animal. Other experiments suggested that the regulation of these processes by the nervous system was mediated by neurosecretory products.

First, several neurohumors were identified in the nervous system by histochemical techniques (epinephrine, norepinephrine, 5-hydroxytryptamine). Secondly, enzymatic mechanisms for the synthesis and destruction of these and other compounds were present (acetylcholinesterase, monoamine oxidase). Thirdly, decrease in the concentration of these substances coincided with inhibition of regeneration. Fourthly, blocking agents of the substances or enzymes inhibited regeneration. Finally, the nervous system was morphologically capable of neurosecretion, since both membrane-bound vesicles and small dense granules appeared to be formed by the Golgi apparatus of nerve cells.

It is hypothesized that growth, differentiation, and attainment of normal form in hydra are regulated by substances secreted by the nervous system. At least one function of the neurosecretory substance may be stimulation of mitosis of interstitial cells and initiation of

their differentiation into other cell types. Presumably, there is genetic control and modification of these processes within both interstitial cells and neurons. Additional evidence concerning the relationship of neurosecretion to differentiation may be obtained from an electron microscopic study of the regenerating hydra to determine the fine structural events, especially those concerned with elaboration and discharge of neurosecretory substance, occurring during regeneration. Finally, isolation of the neurosecretory substance and a study of its effects on normal and regenerating hydra will determine the true biological nature of this material.

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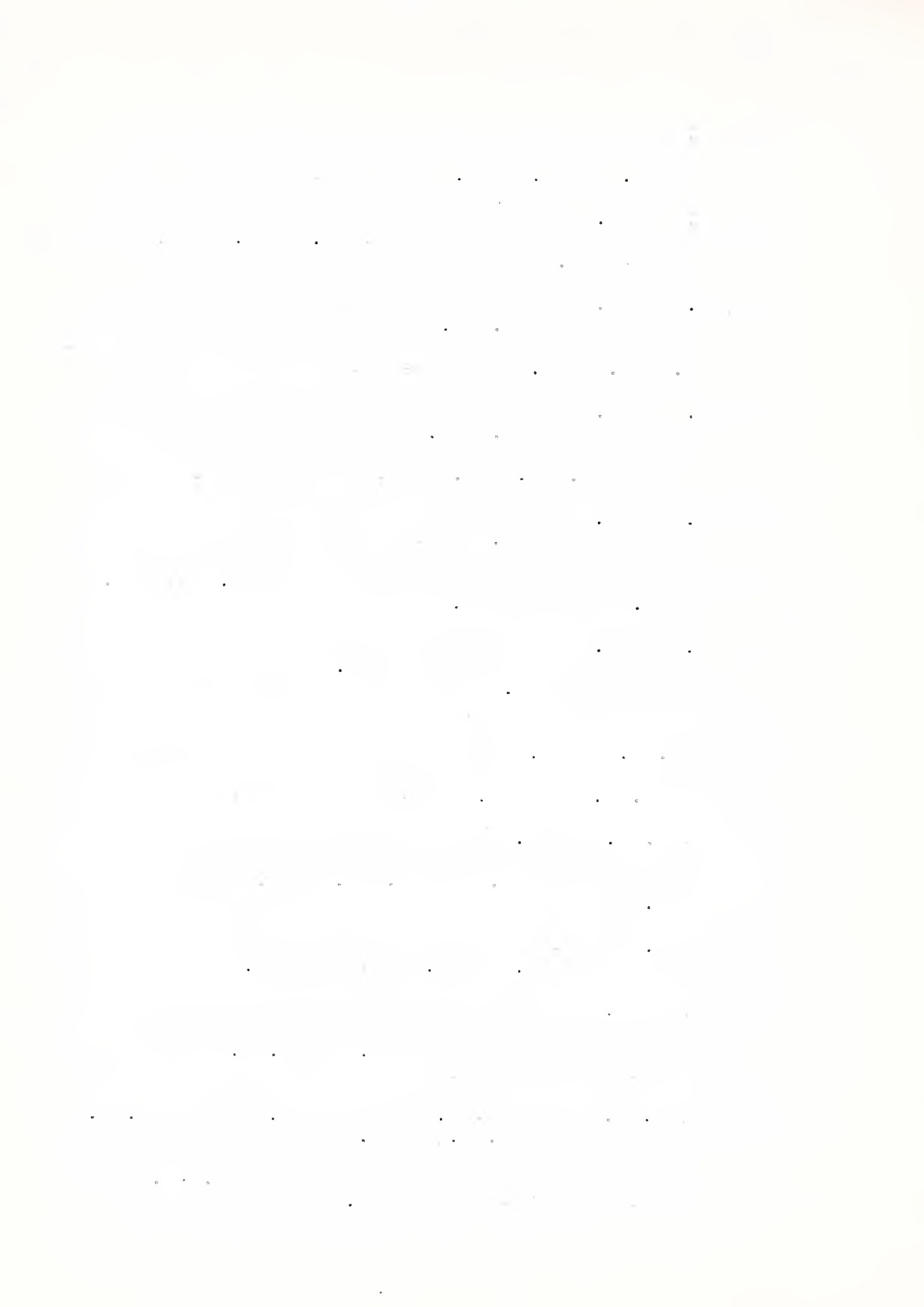
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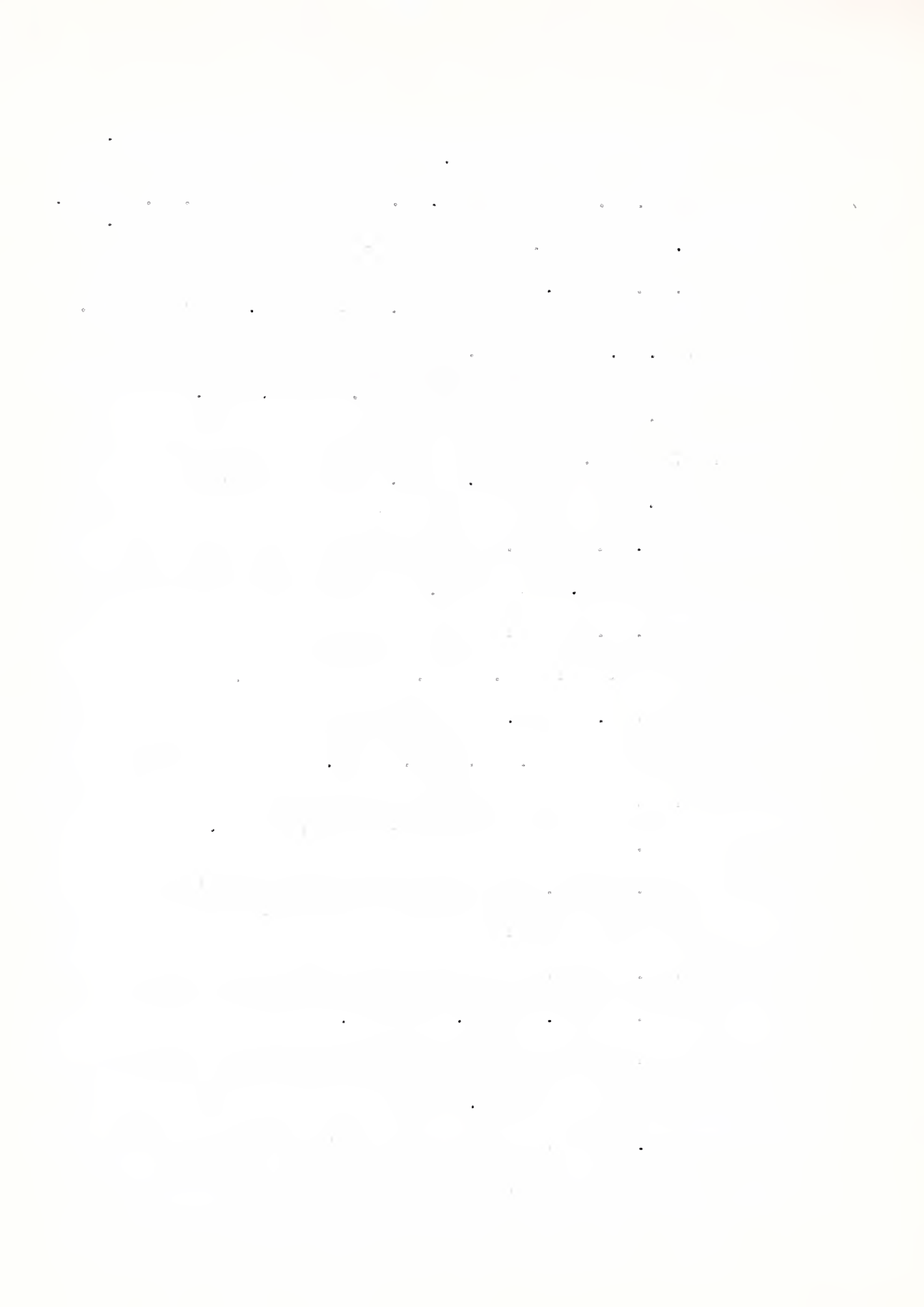
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